

University of Dundee

MASTER OF SCIENCE

Detection of Mouse Hepatitis Virus

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Detection of Mouse Hepatitis Virus

Cheryl Saula Wood

A thesis submitted for the degree of Master of Science in the University of Dundee

November 2015

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List of Abbreviations

BSA	Bovine serum albumin
CAR	Ciliary associated respiratory
DCB	Dichlorobenzene
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme-linked immunosorbent assay
FELASA	Federation of European Laboratory Animal Associations
HEPA	High efficiency particulate arrestance
HOD	Haemoxygenase-1 dual reporter
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IVC	Individually ventilated cages
LCMV	lymphocytic choriomeningitis virus
MFIA	Multiplexed fluorometric immunoassay
MHV	Mouse hepatitis virus
MPV-1	Mouse parvovirus type 1
MNV	Mouse norovirus
MSRU	Medical school resource unit
PCR	Polymerase chain reation
RT-PCR	Reverse transcription polymerase chain reaction
SPF	Specific pathogen free
VAF	Virus antibody-free

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Declaration

I declare that:

- I am the author of this thesis;
- Unless otherwise stated, all references cited have been consulted by me;
- The work of which the thesis is a record has been done by me, and;
- The thesis has not been previously accepted for a higher degree.

Signed:

Cheryl Saula Wood

I declare that the conditions of the relevant Ordinance and Regulations have been fulfilled.

Signed:

Edward Luke Newman

Summary

Mouse hepatitis virus (MHV) is a common infection in a large number of laboratory mouse colonies and is known to interfere with research results. Serology testing is a good method of detecting the historical presence of MHV, however, it cannot detect when the mouse is currently shedding the virus.

The purpose of this study was to develop an in-house Polymerase Chain Reaction (PCR) assay to detect MHV in naturally infected mice using faecal samples. PCR is a highly sensitive test that can detect few copies of virus in a given sample. The PCR was also used to detect the length of time MHV was shedding in C57BL6/J mice.

A number of problems with cross-contamination were encountered and largely overcome. Standard conditions for extracting RNA from faecal pellets, reverse-transcribing it into cDNA and detecting the presence of viral sequences by PCR were developed.

The pattern of viral shedding from “naïve” mice introduced into the animal facility was found to be variable, even between animals housed in the same cage. Not all animals appeared to shed virus at all and, of those that did, some showed more than one cycle of shedding within the observation period.

Shedding was more consistent from post-weaning animals born from matings between members of the introduced cohort, but still with some variability. Not all animals had apparently cleared the virus at the end of the 30-day observation period.

There was generally good correlation between the detection of viral sequences and the ultimate serological status of sample mice, with some notable exceptions.

These results are discussed in the context of using PCR to evaluate the current “viral load” in an animal facility and in developing strategies for elimination of the organism.

1 Introduction

1.1 Microbiological screening in animal units.

Animals are used by scientists in experiments as a vital part of advancement in scientific and medical research. These animals are housed in specialist laboratory units in universities, medical schools and pharmaceutical companies etc. The health status of these animals is very important and a programme of health monitoring is a vital part of the management of the facilities to ensure that the standard of animal health within them is maintained.

1.1.1 Reasons for health screening of animal units

There are a number of reasons why health screening is important in animal units. Some animal pathogens are zoonotic in man and may therefore be serious safety risks. Such organisms, for example lymphocytic choriomeningitis virus (LCMV) and hantaan virus, used to be fairly common in laboratory mice but have now been eradicated from most long-established colonies. Due to their potential impact on human health, however, they are still usually included in standard panels of organisms to be screened for. Proof of the absence of these organisms is often required for the import of laboratory animals into another country, for the same reason.

Some organisms, while no serious threat to human health, can cause significant disease in animal species, and clearly must be excluded. If they are found to be present, then a variety of measures may have to be taken to eliminate them, perhaps even the complete termination of animal holding, fumigation of the facility and re-stocking with known “clean” animals.

Other organisms may have only slight effects on overall animal welfare in normal circumstances but can interfere substantially with scientific experiments, e.g., by causing changes in behaviour, growth rate, relative organ weight and/or immune response. In Mouse Parvovirus type 1 (MPV-1), for example, natural infections are generally asymptomatic, even for neonatal and immunocompromised mice (Smith, Jacoby et al. 1993; Jacoby and Ballgoodrich 1995). However, MPV-1 affects processes linked to cell proliferation. Reported effects include direct modulation and dysfunction of T lymphocytes and altered patterns of rejection of tumor and skin allografts (Baker 1998). Another example of disease effecting animal experiments is Pneumonia virus of

mice. The virus is asymptomatic in euthymic animals (Smith, Carrano et al. 1984), however, causes chronic pneumonia and death in athymic (nude) mice (Richter, Thigpen et al. 1988; Weir, Brownstein et al. 1988). Exposure increases the susceptibility to diabetes induction by streptozotocin in BALB/cByJ male mice (Leiter, Le et al. 1988). It also causes significant decreases in body weights of F344/NCr rats but not of B6C3F1 mice (Rao, Piegorsch et al. 1989).

The use of animals of known biological tendencies is important in ensuring reproducibility of experimental results (Mahler (Convenor), Berard et al. 2014). These sub-clinical infections are particularly insidious as, without regular screening, their presence may otherwise go unnoticed. Their potential to increase variability among animals that otherwise have been chosen to be as similar as possible (e.g., inbred mouse lines) can confound a scientific experiment or, at the very least, greatly increase the number of animals required for a significant result to be obtained. When the purpose of the experiment is, for example, to test the immune response to deliberately administered exogenous agent, the pre-existence of immunologically active organisms can completely confound the data.

With novel lines of genetically modified animals having been created in scientific establishments around the world, the need to move animals between different establishments has grown significantly. Regular health screening can reduce the risk of spreading unsuspected microbial contaminants from one laboratory to another.

1.1.2 Organisms that are screened for

Most laboratories will use a standard panel of organisms to test for, to try to have some uniformity between them. In Europe, the panels most often used were devised by the Federation of European Laboratory Animal Associations (FELASA) (Mahler (Convenor), Berard et al. 2014). Table 1 below contains a list of infectious agents that FELASA recommends are monitored for and the frequency of monitoring, for laboratory mice.

Table 1: Recommended infectious agents to monitor and frequencies of monitoring for laboratory mice (*Mus musculus*)

Viruses	Every 3 months	Annually
Mouse Hepatitis virus	X	
Mouse rotavirus	X	
Murine norovirus	X	
Parvoviruses:	X	
Minute virus of mice	X	
Mouse parvovirus	X	
Theiler's murine encephalomyelitis virus	X	
Lymphocytic choriomeningitis virus	X	
Mouse adenovirus type 1 (FL)		X
Mouse adenovirus type 2 (K87)		X
Mousepox (ectromelia) virus		X
Pneumonia virus of mice		X
Reovirus type 3		X
Sendai virus		X
Bacteria		X
<i>Helicobacter</i> spp. If positive, speciation for <i>H. hepaticus</i> , <i>H. bilis</i> and <i>H. typhlonius</i> is recommended		
<i>Pasteurella pneumotropica</i>	X	
Streptococci β -haemolytic (not in group D)	X	
<i>Streptococcus pneumonia</i>	X	
<i>Citrobacter rodentium</i>	X	
<i>Clostridium piliforme</i>		X
<i>Corynebacterium kutscheri</i>		X
<i>Mycoplasma pulmonis</i>		X
<i>Salmonella</i> spp.		X
<i>Streptobacillus moniliformis</i>		X
Parasites		X
Endo- and ectoparasites (reported to the genus level)		
Additional agents*	X	
Viruses:		
Hantaviruses		
Herpesviruses (mouse cytomegalovirus, mouse thymic virus)		
Lactate-dehydrogenase elevating virus		
Polyomaviruses (mouse polyomavirus, K virus)		
Bacteria and fungi:		
Cilia-associated respiratory bacillus		
<i>Klebsiella oxytoca</i> , <i>Klebsiella pneumonia</i>		
Other <i>Pasteurellaceae</i> [†]		
Pneumocystis murina		
Pseudomonas aeruginosa		
Staphylococcus aureus		
Others as necessary		

All agents listed should be reported if found in diagnostic examinations irrespective of when they are found.

*Testing for these agents is optional and should be pursued if there is a specific need. Frequency of testing will depend on local circumstances.

[†]We acknowledge that the inclusion of the *Pasteurellaceae* family is controversial (this is a common bacteria which can cause respiratory disease, but usually doesn't. It is not definitive that they are always pathogenic or interfere with experiments). Screening for the family can be conducted should the facility wish, and the difficulty of some commercial kits to correctly identify *Pasteurella pneumotropica*, as well as the fluidity of the correct phenotypic classification, should also be acknowledged. Table taken from (Mahler (Convenor), Berard et al. 2014)

Commercial suppliers usually offer animals that are rigorously monitored. As just one example, Charles River carries out daily surveillance and careful monitoring of reproductive parameters. Fortnightly tests are carried out, of environment samples by PCR and groups of animals by serology, for the most common agents. Every four weeks, TaqMan® PCR is used to screen 10 samples from environmental sites in the barrier rooms. i.e., air exhaust grates and bedding disposal equipment. They also perform serologic monitoring of 16 animals from each Specific Pathogen Free (SPF) mouse production room, testing the most prevalent viruses.

In addition, every 13 weeks they evaluate 12-16 animals from three different age groups from each SPF area by a comprehensive health monitoring protocol including serology, bacteriology, parasitology, gross pathology and PCR for all *Helicobacter* spp.

Each colony is also assessed annually by PCR for many common viruses, bacteria, fungi and parasites.

The above information was obtained from Charles River's technical sheet "Routine Health Monitoring of Charles River Rodent Barrier Production Colonies in Europe and North America". Colony health status reports are updated weekly on their website which includes any new colony status results. Table 2 is also taken from Charles River's technical sheet and gives details regarding agents, frequency of testing and methods used. It tests for all the infectious agents recommended on the FELASA list (Table 1) and some additional organisms.

These "SPF / VAF (virus antibody-free) animals are of the usual status purchased by the University of Dundee and many other scientific establishments. However, Charles River, Harlan and other suppliers will also sell animals that have been bred in isolation and for which even more microbiological screening is done.

Table 2: Charles River table of agents tested for, method and frequency of testing

charles river

EUROPE SPF/VAF Barrier Room
EUROPE

The Jackson Laboratory

Charles River Laboratories Kent
Location
UK A52 Mice

Health Status
SPF / VAF

Summary Item	Method	Primary Lab	Test Date	Most Recent		Past 18 Months	
				Positive / Tested		Positive / Tested	
Viruses							
Minute Virus of Mice (MVM) ad	MFIA	RADS EU	13-Mar-2015	0 / 16		0 / 304	
Mouse Parvovirus (MPV) ad	MFIA	RADS EU	13-Mar-2015	0 / 16		0 / 304	
Mouse Hepatitis Virus (MHV) ad	MFIA	RADS EU	13-Mar-2015	0 / 16		0 / 308	
Mouse Norovirus (MNV) ad	MFIA	RADS EU	13-Mar-2015	0 / 16		0 / 304	
Theriler Virus (TMEV-GDVII) ad	MFIA	RADS EU	13-Mar-2015	0 / 16		0 / 304	
Mouse Rotavirus (EDIM) ad	MFIA	RADS EU	13-Mar-2015	0 / 16		0 / 304	
Sindai (SEND) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
Reovirus 3 (REC3) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
Pneumonia Virus (PVM) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
Mouse Thymic Virus (MTV) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Mouse Cytomegalovirus (MCMV) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Hantaan Virus (HANT) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Lymph chortomening (LCMV) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
Mouse Adenovirus (MAV1&2) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
K virus (K) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Ectromelia (ECTRO) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
Polyoma Virus (POLY) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Lactate Dehydrogenase (LDV) ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Bacteria							
Tyzer's Disease (CPIL) ae	Exam	RADS EU	19-Feb-2015	0 / 12		0 / 92	
Bordetella bronchiseptica be	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Citrobacter rodentium ae	Culture	RADS EU	19-Feb-2015	0 / 4		0 / 60	
Corynebacterium kutscheri ae	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Mycoplasma pulmonis ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
Pasteurella pneumotropica be	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Pasteurella multocida ae	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 48	
Salmonella spp ae	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Streptobacillus moniliformis ae	PCR	RADS EU	19-Feb-2015	0 / 12		0 / 108	
Beta Streptococcus - Grp A ae	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 48	
Beta Streptococcus - Grp G ae	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Streptococcus pneumoniae be	Culture	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Helicobacter hepaticus ae	PCR	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Helicobacter bilis be	PCR	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Helicobacter - other spp ae	PCR	RADS EU	19-Feb-2015	0 / 8		0 / 56	
CAR Bacillus ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 72	
Parasites							
Ectoparasites ae	Exam	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Helminths ae	Exam	RADS EU	19-Feb-2015	0 / 8		0 / 64	
Enteric Pathogenic Protozoa ae	Exam	RADS EU	19-Feb-2015	0 / 8		0 / 48	
E. cuniculi ae	MFIA	RADS EU	19-Feb-2015	0 / 8		0 / 56	
Lesion Observed							
Gross Exam: bg	Exam	RADS EU	19-Feb-2015	0 / 12		0 / 92	

LTM

Page 1 of 2

Reported on Friday, March 20, 2015 at 5:43

charles river

EUROPE SPF/VAF Barrier Room
EUROPE

The Jackson Laboratory

Charles River Laboratories Kent
Location
UK A52 Mice

Health Status
SPF / VAF

Summary Item	Method	Primary Lab	Test Date	Most Recent		Past 18 Months	
				Positive / Tested		Positive / Tested	
Lesion Observed							
Gross Exam: bg	Exam	RADS EU	19-Feb-2015	0 / 12		0 / 92	
* dashes indicate not tested during specified period.							
HOUSED STRAINS							
	Inbred BALB/CANN C57BL/6J DBA/1J	Hybrid CB6F1					

Legend:
RADS EU = Research Animal Diagnostic Services Europe Lyon, France
RADS US = Research Animal Diagnostic Services United States Wilmington, Massachusetts

COLONY POLICY FOR POSITIVE RESULT: a = immediate termination;
b = planned future recycle of the colony; c = no action.

TESTING SCHEDULE:
d = screened every four or five weeks; e = screened quarterly;
f = screened annually; g = screened every 12 or 13 weeks
by necropsy examination.

* Sampling date/Results validation could take until 3 weeks.

A52 CRUK Mice SPF

LTM

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Reported on Friday, March 20, 2015 at 5:43

1.1.3 *Methods for screening*

Traditionally, rodent viruses have been detected indirectly, by analysing blood samples (serology) for the presence of anti-viral antibodies. Screening methods include Enzyme Linked Immunosorbent Assay (ELISA) and Immunofluorescence Assay (IFA). Viral antigen is immobilised on a plate or bead substrate and incubated with the blood sample. After washing, the substrate is then incubated with a fluorescence- or enzyme-linked secondary antibody that binds to mouse antibodies. If the plate or bead becomes associated with above-background levels of fluorescence or enzyme activity, then the presence of antibodies to the virus is inferred and therefore it is concluded that, at some point in the past, the animal has been exposed to the virus (Figure 1). This is performed by which one microbial antigen-antibody reaction is measured per well. Multiplexed Fluorometric ImmunoAssay (MFIA®), in comparison, is performed as a *multiplexed* assay. Since the microspheres come in 100 distinct colour sets, as many as 100 different assays can be performed simultaneously in a single microplate well. This innovation decreases the amount of serum, reagents and disposables required for routine testing while increasing the amount of information obtained from a single test well (Wunderlich, Dodge et al.) (Figure 2).

Enzyme-linked Immunosorbent Assay

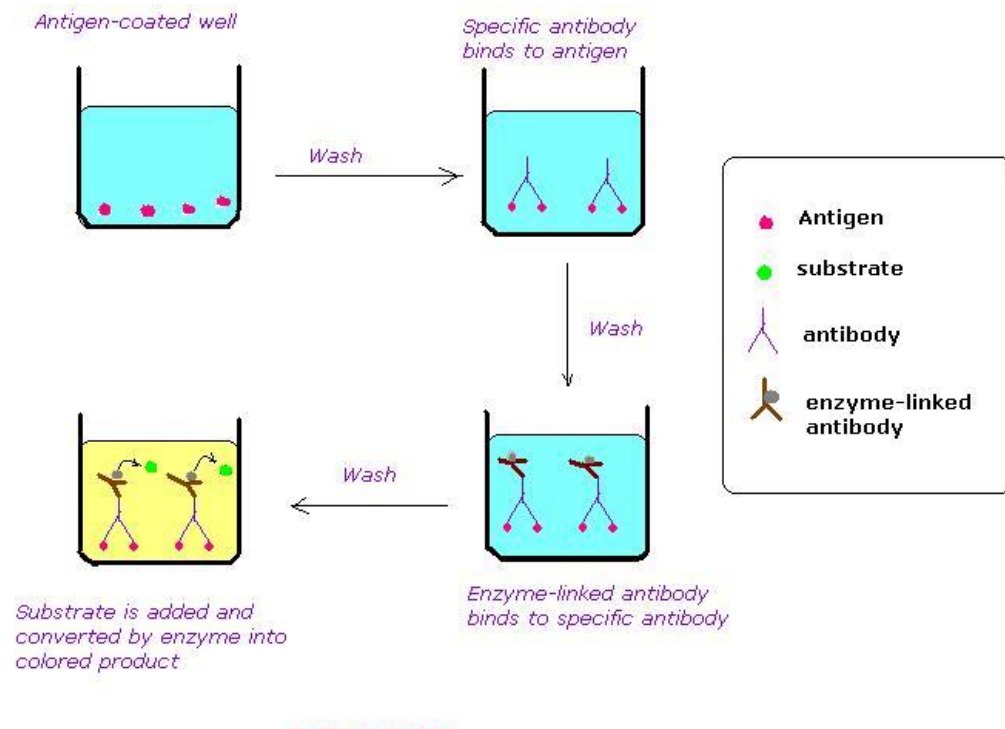


Figure 1: Example of steps in ELISA test to determine if a particular antibody is present

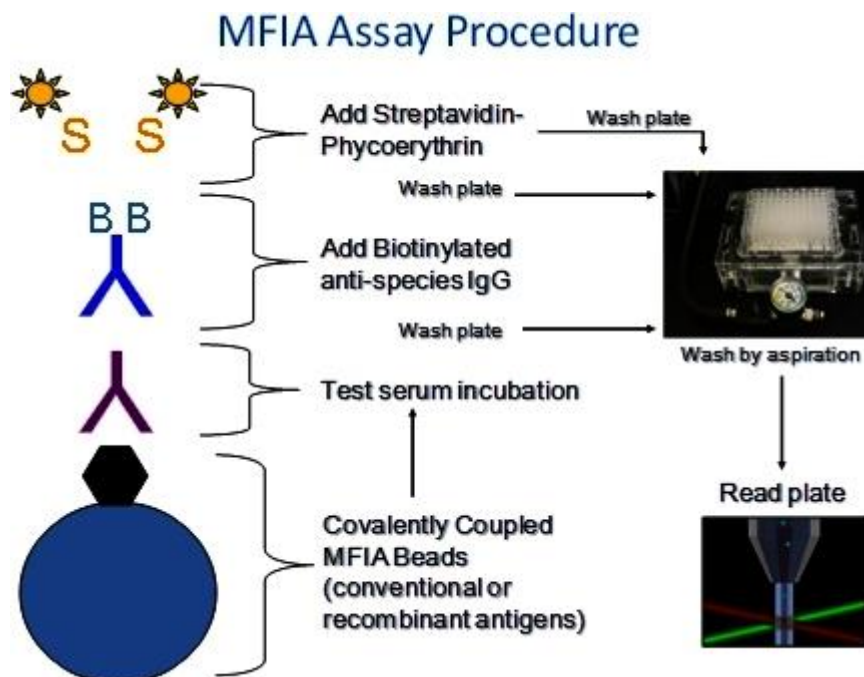


Figure 2: MFIA Procedure. The xMAP-based MFIA is a suspension microarray which utilizes color-coded polystyrene 5.6 micron beads to which antigens (or controls) are covalently linked. Since the beads come in 100 distinct color sets, as many as 100 different assays can be performed in a single well. Assay steps are performed in filter-bottom microtiter plates so that beads can be washed by aspiration on a vacuum manifold. Reactions are read with the Luminex xMAP 100 fluorometer. The intensity of phycoerythrin fluorescence is reported as a median fluorescence index (MFI) (Wunderlich, Dodge et al.)

Bacteria are usually screened for by direct culture and intestinal parasites by microscopic examination of a representative number of slides (looking for the organism itself, and/or the oocyst stage).

Of course, serology does not work well in immune-deficient animals, nor does it allow one to conclude which animals are currently infected and actively shedding the virus. It requires expensive equipment and proprietary reagents to perform and is therefore normally sub-contracted to a specialist laboratory, though with inevitable delays and concerns about quality control.

More recently, Reverse Transcriptase-coupled Polymerase Chain Reaction (RT-PCR) methods have been developed that detect the viral nucleic acid directly. Viral RNA is first extracted, usually from mouse faecal samples (as MHV is usually an infection of the gastrointestinal tract, such samples should be a source of viral RNA) (Figure 3). DNA that is complementary to a specific target area within the RNA (i.e. polymerase gene, N gene, M gene) is then created by reverse transcribing it using a suitable oligonucleotide primer and reverse transcriptase. A region of the newly created DNA is then amplified by Polymerase Chain Reaction (PCR) (Figure 4).

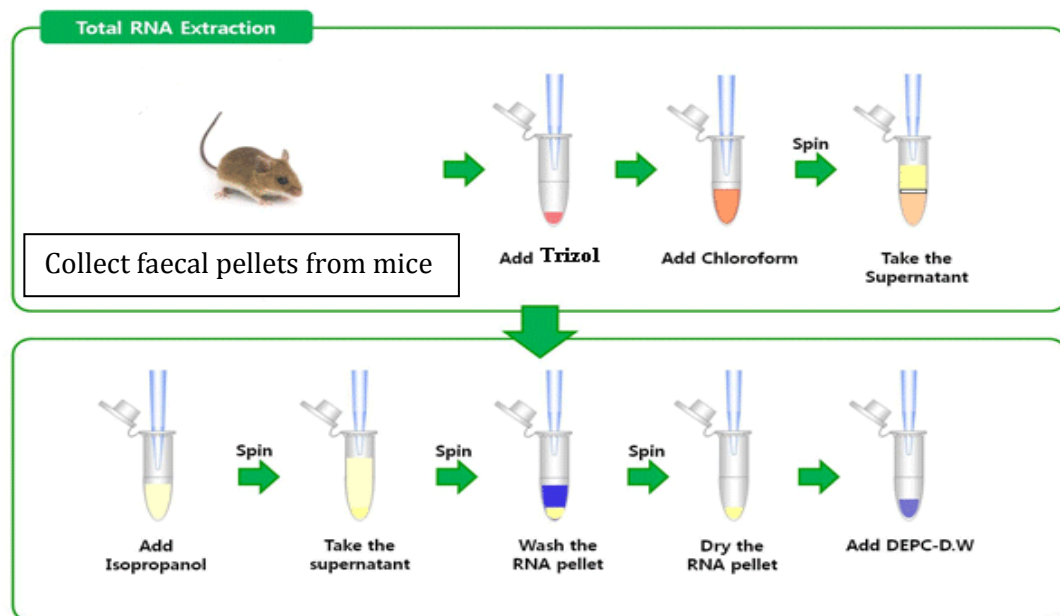


Figure 3: Total RNA extraction from mouse faecal pellets.

Figure shows a typical RNA extraction using the Trizol method, adapted from www.bioneer.com

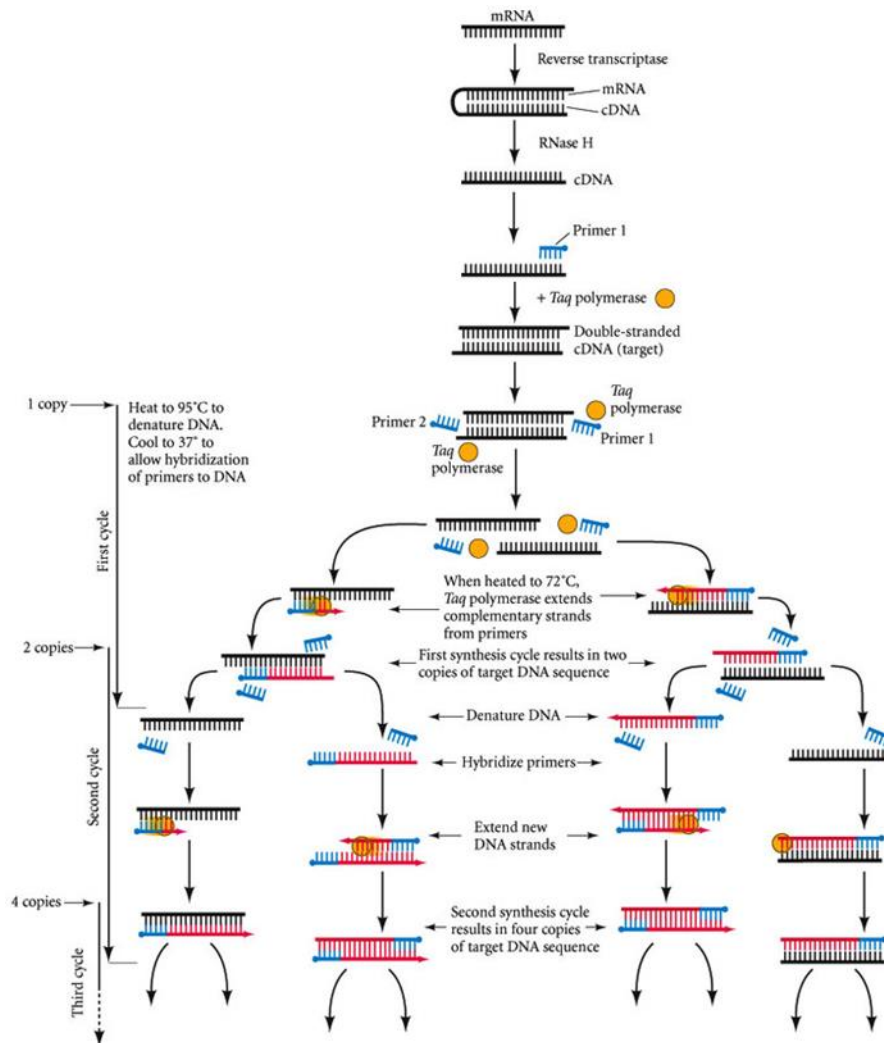


Figure 4: Example of a Reverse-Transcriptase Polymerase Chain Reaction

Flow of the process involved in a typical RT-PCR, taken from (Gilbert 2013)

Table 3 below shows an example of a typical health screen carried out by a commercial diagnostic facility on sample mice in the Medical School Resource Unit (MSRU) at Ninewells Hospital, Dundee. It lists what is being tested for, results of virology, microbiology, pathology, parasitology and the type of assay used for testing, positive results and number of animals tested. Compared with the FELASA screen, it tests for all the recommended agents plus some extra ones, i.e. mouse polio virus, ciliary-associated respiratory (CAR) Bacillus, *Corynebacterium kutscheri*, *Encephalitozoon cuniculi* and enteric protozoa. This particular report does not, however, indicate the frequency of testing. On this specific occasion, serologically positive tests for mouse

norovirus and enteric protozoa were obtained. However, the historical record also reveals the presence of mouse hepatitis virus (MHV) and endoparasites (almost certainly helminths),

It is not practicable to test every one of many thousand mice in a facility, and therefore a system of representative sampling must be adopted. In the Ninewells unit, animals are sampled randomly from a number of the rooms. As the mice are housed in conventional grid-top cages, which are washed in a communal area (dirty cages are brought into the cage washing area through a dirty side, the dirty bedding is scrapped out and the cages are then placed in a conveyor belt tunnel washer, they are then filled with fresh sawdust and exit through a clean end) it is to be expected that an organism, if present at all, will manifest itself in samples from several rooms.

Where mice are housed in individually ventilated cages, to isolate them from the microbiological environment of the main facility, (the cages are autoclaved before use together with bedding and food) the representative sampling is not possible, as each cage functions as an isolator. In this case "sentinel" animals are exposed to soiled bedding from these cages on a regular basis and then it is these animals that are tested.

Table 3: Example of typical health screen performed on mice from Medical School Resource Unit, at Ninewells Hospital, Dundee

Location: DUNDEE NINEWELLS Main Unit

Sponsor: Dundee Univ Ninewells (J.Mcleod)

Product: SPF Mice

Reported: Tuesday, December 29, 2009 at 7:42

Most Recent

Past 24 Months

Summary Item	Primary Assay	Year-Week *	Positive/Tested	Positive/Tested
Virology				
Sendai Virus (SEND)	MFIA/ELISA	2009-51	0/ 6	0 / 140
Pneumonia Virus of Mice (PVM)	MFIA/ELISA	2009-51	0/ 6	0 / 140
Mouse Hepatitis Virus (MHV)	MFIA	2009-51	0/ 6	42/ 150
Minute Virus of Mice (MVM)	MFIA	2009-51	0/ 6	0/ 140
Mouse Parvovirus (MPV)	MFIA	2009-51	0/ 6	0/ 140
Murine Norovirus (MNV)	MFIA	2009-51	2/ 6	43/ 140
Mouse Polio Virus (TMEV (GDVII))	MFIA	2009-51	0/ 6	0/ 140
Reovirus (REO)	MFIA/ELISA	2009-51	0/ 6	0/ 140
Mouse Rotavirus (EDIM)	MFIA	2009-51	0/ 6	0/ 141
Lymphocytic Choriomeningitis Virus	MFIA/ELISA	2009-51	0/ 6	0/ 140
Edromelia Virus (ECTRO)	MFIA	2009-51	0/ 6	0/ 140
Mouse Adenovirus (MAV)	MFIA/ELISA	2009-51	0/ 6	0/ 140
Mouse Cytomegalovirus (MCMV)	MFIA	2009-51	0/ 6	0/ 140
Mouse Pneumonitis Virus (K)	MFIA	2009-51	0/ 6	0/ 140
Polyma Virus (POLY)	MFIA	2009-51	0/ 6	0/ 140
Hantaan Virus (HANT)	MFIA	2009-51	0/ 6	0/ 140
Mouse Thymic Virus (MTLV)	IFA	2009-25	0/42	0/ 132
Microbiology				
<i>B.bronchiseptica</i>	Culture	2009-51	0/ 6	0/ 103
<i>CAR Bacillus</i>	MFIA/ELISA, PCR	2009-51	0/ 6	0/ 140
<i>C.rodentium</i>	Culture	2009-51	0/ 6	0/ 103
<i>C.kutscheri</i>	Culture	2009-51	0/ 6	0/ 103
<i>H.bilis</i>	PCR	--		
<i>H.hepaticus</i>	PCR	--		
<i>Helicobacter sp.</i>	PCR	--		
<i>Klebsiella spp.</i>	Culture	2009-51	0/ 6	0/ 103
<i>M.pulmonis</i>	MFIA/ELISA, PCR	2009-51	0/ 6	0/ 140
<i>Pasteurellaceae</i>	Culture	2009-51	0/ 6	0/ 103
<i>Ps.aeruginosa</i>	Culture	2009-51	0/ 6	0/ 103
<i>Salmonella sp.</i>	Culture	2009-51	0/ 6	0/ 103
<i>Staph.aureus</i>	Culture	2009-51	0/ 6	0/ 103
<i>S.moniliformis</i>	Culture	2009-51	0/ 6	0/ 103
<i>Beta Strep. Spp</i>	Culture	2009-51	0/ 6	0/ 103
<i>Strep.pneumonia</i>	Culture	2009-51	0/ 6	0/ 103
Pathology				
Gross Exam	Necropsy	2009-51	0/ 6	0/ 103
Parasitology				
<i>E.cuniculi</i>	MFIA/ELISA	2009-51	0/ 6	0/ 140
Ectoparasites	Exam	2009-51	0/ 6	0/ 103
Endoparasites	Exam	2009-51	0/ 6	1/ 103
Enteric Protozoa	Exam	2009-51	2/ 6	13/ 103

* dashes indicate not tested during specified period.

1.1.4 History of the Medical School Resource Unit

The Medical School Resource Unit (MSRU) at Ninewells Hospital was built with the hospital in the early 1970s, and over the past 40 years has been host to a wide range of large and small animal species, ranging from pigs and sheep to small rodents and frogs. Currently, the Unit has permanent holdings of mice and rats only. There are approximately 20 animal holding rooms. The majority of the animals are housed in conventional grid-top cages (Figure 5), with no significant microbiological barrier between cages in the same room and only a limited barrier (created by operating the holding rooms at negative air pressures with respect to the common corridors) between one room and the next. All the used cages are cleaned and re-stocked in a common cage-washing area. Some mice are kept in individually ventilated cages (IVCs) (Figure 5), in which the air is delivered to and extracted from each cage via HEPA filters. Each IVC therefore operates as a micro-isolator, with a very high barrier even between cages on the same rack. Some animals are kept in positively pressured IVCs primarily to keep them free from micro-organisms that might be present in the conventionally-housed stocks. Others are kept in specially sealed negative-pressure systems, usually because they have been imported from elsewhere and they are to be used as donors for the collection of sperm or embryos for rederivation into a “clean” unit elsewhere.

A



B



Figure 5: Examples of cage types used in Medical School Resource Unit, Ninewells Hospital

A - Shows typical example of open-top grid cage (North Kent Plastics M1); B - Shows a typical individually ventilated cage (Techniplast)

1.1.5 *Micro-organisms found in the MSRU*

The mouse stocks in the unit, which make up the great majority of the animals and most of which are in breeding programmes, routinely test positive for mouse norovirus (MNV), mouse hepatitis virus (MHV), *Helicobacter* spp., *Pasteurella* spp., and a variety of intestinal parasites including *Syphacia* spp., (pinworm) and *Entamoeba*. There may also be some commensal bacteria that are shared with humans, such as *Staphylococcus* spp., and *Klebsiella* spp. Of these agents, the MNV, *Pasteurella*, commensals and *Entamoeba* are not really regarded as being threats to human or animal welfare, nor are they likely to confound the types of research undertaken in the resource unit. While mouse norovirus is genetically related to the human virus responsible for outbreaks of diarrhoea and vomiting (most famously on cruise ships and in hospital wards), it is neither transmissible to humans nor does it cause outward signs of disease in mice unless its host is completely immune-deficient (Karst, Wobus et al. 2003), i.e. without either innate or acquired immunity. Pinworm could be a research problem, as it has the potential to cause low-grade inflammation of the gut (Taffs 1976), and can effect experimental results. Unusual autoimmune responses were linked to infestation with a common rodent pinworm (Agersborg, Garza et al. 2001). But again this has not been found to be a serious problem in practice. While pinworm is easily killed by feeding the animals on a diet containing a benzimidazole anti-helminthic drug, e.g., fenbendazole, the oocyst stage of the organism's life-cycle is highly resistant to most disinfectants, dehydration, autoclaving and other cleaning regimes. Re-infection after drug treatment is thus a very common finding. Indeed, in the late 2000s, while the MSRU was being refurbished, a determined effort was made to eradicate this organism but, after two years of apparently negative screens, it was soon detected once more.

MHV, on the other hand, has a history of causing serious disease in infant and immunodeficient mice. In infant mice clinical signs include diarrhea, poor growth and death and immunodeficient mice develop a wasting syndrome characterized by severe generalized disease and eventual death (Uetsuka, Nakayama et al. 1996). MHV is also capable of confounding scientific data even when its outward effects are much less significant. Some examples of interference with research include:

Contamination of transplantable tumours (Wunderlich, Dodge et al.)

Rejection of human xenografts (Kyriazis, DiPersio et al. 1979)

Altered response to chemical carcinogens (Barthold 1986a)

Immunodepression and immunostimulation depending on the time of infection

(Virelizier, Virelizier et al. 1976)

Decrease of the incidence of diabetes in non-obese diabetic mice (Wilberz, Partke et al. 1991)

1.1.6 The accidental introduction of MHV into the MSRU

For about the last twenty years, the MSRU has operated an apparently robust policy on the import of animals from other establishments. In particular, documentation has always had to be produced to demonstrate that the facilities from which the animals are to come are apparently free from all significant micro-organisms, other than those that are already known to be present. The commercial suppliers of laboratory rodents match this requirement as a matter of routine. However, not all imports from other research organisations have been approved.

In 2004, the resource unit took delivery of mice from another academic establishment, which had provided the necessary documentation in advance. However, between the last screen being done at that establishment and the mice arriving in the MSRU, MHV contamination entered. As soon as that establishment realised the fact, the manager of the MSRU was contacted and samples for MHV analysis were immediately sent away for testing. Initial results were negative but, because of the potential seriousness of the situation, animals were re-screened and positive results were reported. As the imported animals had been housed in conventional grid-top cages, there was little hope that the contamination was confined to only that room and, indeed, more extensive testing revealed the organism to be present throughout the resource unit.

1.1.7 Potential strategies for eradication

The definitive method for eradicating MHV is to kill all the susceptible animals, fumigate the entire facility and then re-stock. However, as the particular strain did not appear to be associated with some of the more severe effects associated with MHV infection, and this strategy would have halted all research work within the resource unit and eliminated some valuable mouse lines that would not easily be found from elsewhere, this option was rejected by the scientific community, the veterinary surgeon and the unit management.

Since it appeared that infection was readily survivable, an alternative “firewall strategy” was considered in some detail. The principle is to halt the import of all non-infected animals and the breeding of all lines already present in the unit. Once every

susceptible animal has become infected and then eliminated the virus (though it will now be sero-positive for anti-viral antibodies), the infection may be considered to have “burned itself out”. The period of time over which these draconian measures must be in place has been variously estimated as between six and 12 weeks. However, one would have to be absolutely certain that the virus had gone before allowing breeding to restart and for animals to be ordered from the usual suppliers once more. While it might have preserved the work of scientists who did not need to breed any animals, the great majority of the mouse lines in the unit were in breeding programmes and therefore the risk of losing valuable lines through not being able to produce the next generation would have been highly significant, particularly if the firewall had to be in place for a protracted period.

Success or failure of the firewall strategy would have depended on knowing the life-cycle of the precise viral strain circulating in the unit, so as to be able to calculate the “burnout period”. It would also have required access to a test for the presence of the virus itself (rather than the historical data produced by serology), that could be applied very quickly and economically to large numbers. PCR-based tests were in their infancy at the time so it would not have been feasible to deploy them. Because of the lack of robust evidence that the particular viral strain was causing any welfare issues or confounding any of the specific research projects current in the unit, again it was decided not to attempt the firewall strategy but to allow the infection to become enzootic in the conventionally-housed mouse colonies. However, some imported lines have been housed in IVCs successfully, without their apparently becoming infected.

1.1.8 Current practice of the animal unit

The animal unit at Ninewells Hospital does not currently accept mice from any other academic establishment into conventional housing without first isolating them in IVCs and determining the precise microbiological status of the animals actually supplied. This practice is not a general distrust in the management of academic facilities, it simply acknowledges that, as part of their normal business, they frequently import animals from other sites and therefore are at risk of the type of event that befell the MSRU.

1.2 Mouse hepatitis virus

1.2.1 *Transmission of the virus*

MHV is one of the most common viruses found in laboratory mouse colonies (Homberger, Zhang et al. 1998) and there have been numerous reports documenting its potential to interfere with research in both immunocompromised and immunocompetent mice. MHV may be transmitted through aerosols, fomites (fur, dander, etc.) and direct contact (Wilberz, Partke et al. 1991). The virus is highly contagious, although not persistent in the environment. MHV may also contaminate cell cultures and transplantable tumours (Nicklas, Kraft et al. 1993). Infection in immunocompetent mice can be asymptomatic (Compton, Barthold et al. 1993), as it appears to be in the MSRU. Expression of the disease depends on the age, genotype, sanitary status, and experimental status of the mouse and the tropism and virulence of the infecting strain of the virus (Barthold, Beck et al. 1993). In enzootic colonies, the infection survives amongst newly weaned animals, as these have lost the maternal passive immunity but have not yet developed a robust anti-viral immune reaction themselves. (Baker 1998). “Nude” athymic mice (which have essentially no adaptive immune response) infected with MHV develop a wasting disease with high morbidity under natural conditions (Kunita, Terada et al. 1992).

1.2.2 *Detection of virus*

Traditionally, MHV screening has relied on the detection of anti-viral antibodies in a sample of blood (serology, as above), usually carried out by commercial companies which can be expensive. Often, the sampled mice are “sentinels”, i.e. animals originally known to be negative for such antibodies but which have been exposed to soiled bedding from the cages being screened. This allows many cages to be screened by the analysis of relatively few animals. Presence of anti-viral antibodies (extracted from MHV infected cells) indicates that the animal has been exposed to MHV in the past, but not necessarily that it has an active infection. Serology assays have the disadvantage of requiring a waiting period of several weeks for an immune response to develop (seroconversion). Newly infected animals, or those in which the infection has reached its peak, will not necessarily have seroconverted yet and may be reported as negative. Many testing laboratories require that the tested animals have been in potential contact with the relevant organism for six weeks or longer, to enhance the chances that they have indeed become infected and mounted a strong immune response. Serological which is then run on an electrophoresis gel (typically 1.5% agarose) to check if the

testing is not reliable for screening immunodeficient mice directly (Casebolt, Qian et al. 1997).

More recently, nucleic acid-based tests for many of the organisms on the standard panels have been developed, as described above.

Primers that will hybridise to opposite strands either side of the region to be amplified (i.e. Polymerase gene, N gene, M gene) are mixed with the DNA in a suitable chemical buffer solution, containing deoxy-ribonucleoside triphosphates (dNTPs, the building blocks from which the DNA polymerase synthesizes a new strand of DNA) and a DNA polymerase. Chemicals such as dimethyl sulfoxide (DMSO), which inhibits secondary structures and improves amplification of GC rich regions which further increase the amplification of the targeted sequence, and Bovine Serum Albumin (BSA) which aids PCR in the presence of inhibitors such as tannic acids (found in foods), or extracts from faeces, are often added as well. In the first cycle, the two-stranded DNA “template” is denatured and then allowed to hybridise to the primers. Extension from these sites then produces four strands from the original two. In subsequent cycles, this repeats, resulting in an approximate doubling of the amount of DNA corresponding to this defined region, eventually resulting in enough product for visualisation on an electrophoresis gel.

The denaturation step involves heat, but the use of a heat-stable DNA polymerase (e.g., Taq, derived from the thermophilic bacterium *Thermophilus aquaticus*) means that many cycles can take place without the need to add new reagents to the tube. A thermal cycler is used to heat and cool the samples to the temperatures required for each step of the reaction. An initial denaturing is carried out by heating the sample to a high temperature for approximately 1-3 minutes, this is essential to completely separate the strands of the template DNA by breaking the hydrogen bonds between them, at the beginning of the PCR run to ensure efficient utilization of the template during the first amplification cycle. The reaction then starts with a denaturation step whereby the sample is heated to a high temperature for 20-30 seconds. The temperature is then lowered to a medium temperature for 20-30 seconds to allow annealing of the primers to the single stranded DNA. The temperature is then increased again for the elongation step of the PCR. At this stage the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand. These steps are then repeated 25-40 times in order to produce thousands of copies of the DNA fragment. A final elongation time is then performed during the last cycle for 5-15 minutes to ensure any remaining single-stranded DNA is fully extended. A dye is then added to the resulting product

amplified fragment does indeed have the expected size. A DNA ladder which contains DNA fragments of known size is also added to the gel to compare with the size of the sample.

The complete PCR procedure is now commonly used in research laboratories for a variety of purposes including the detection and diagnosis of infectious diseases. In contrast to serological assays, PCR assays can indicate which animals are shedding virus at the time of sampling, but not those that may have been infected in the past but are currently free from the virus. The turnaround time is also much faster than serology testing.

1.2.3 Virus structure and life cycle

MHV (figure 6) belongs to the coronaviridae family of enveloped viruses. It has a 31kb single stranded positive RNA genome (Lai 1990) which is divided into 7 genes (figure 7). “Positive” indicates that the viral RNA can be translated directly into proteins in the infected cell. The first gene (polymerase) is 22kb long, a non-structural protein encompassing more than two-thirds of the length of the genome and believed to encode the RNA-dependent RNA polymerase (Pachuk, Bredenbeek et al. 1989). The functions of the other non-structural proteins remain unknown and each of them has been found missing in at least one virus strain suggesting that they are not essential for MHV replication (Yokomori and Lai 1991). Gene 2 encodes the 30kD non-structural protein, gene 2-1 codes for the haemagglutinin/Esterase (HE) protein which is present in some MHV strains (Homberger 1997). In MHV-A59, however, the HE gene is not expressed: its dedicated mRNA, mRNA 2-1, is not produced because of a mutation in the “transcription-regulating sequence” (de Groot 2006). Gene 3 encodes the spike (S) protein. It is usually cleaved into two subunits, S1 and S2 (Sturman, Ricard et al. 1985). S1 is responsible for receptor binding and S2 for membrane fusion (Bosch, van der Zee et al. 2003). Gene 4 encodes a 15kD non-structural protein. Gene 5 contains two open reading frames 5a and 5b. 5a encodes a 13 kD non-structural protein and 5b the small membrane protein (E) (Homberger 1997). The small membrane protein (E) may play a role in either uncoating or assembly of the virus (Yu, Bi et al. 1994). Gene 6 encodes the membrane (M) protein. It is the most abundant envelope protein component having essential functions in virus assembly (Raamsman, Locker et al. 2000). Gene 7, the nucleoprotein (N) is a major structural component of virions that associates with genomic RNA to form a long, flexible, helical nucleocapsid. Interaction with the M protein leads to the formation of virus particles. It may play roles in viral transcription, translation, and/or replication (Ning, Lakatoo et al. 2003).

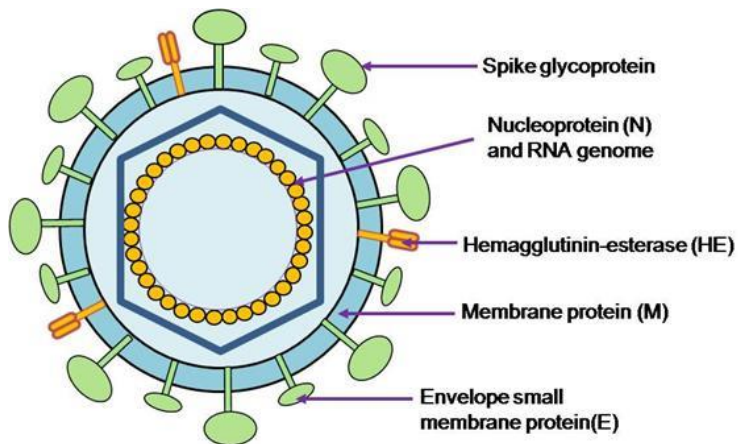


Figure 6: Structure of Mouse Hepatitis Virus

Taken from www.lookfordiagnosis.com

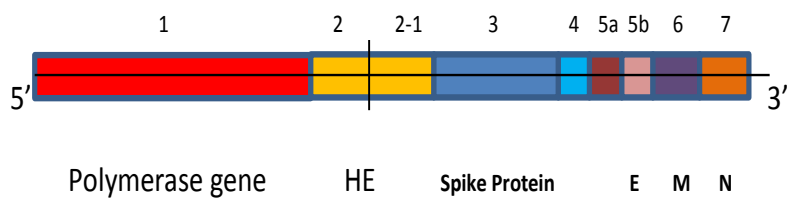


Figure 7: RNA Genome

1. Polymerase gene, 2. Non-structural protein, 2-1. Haemagglutinin/Esterase protein (some MHV strains), 3. Spike (S) protein, 4. Non-structural protein, 5a. Non-structural protein, 5b. Small membrane protein (E), 6. Membrane protein (M), 7. Nucleoprotein (N)

The virus replicates in the cytoplasm of infected cells. (Figure 8).

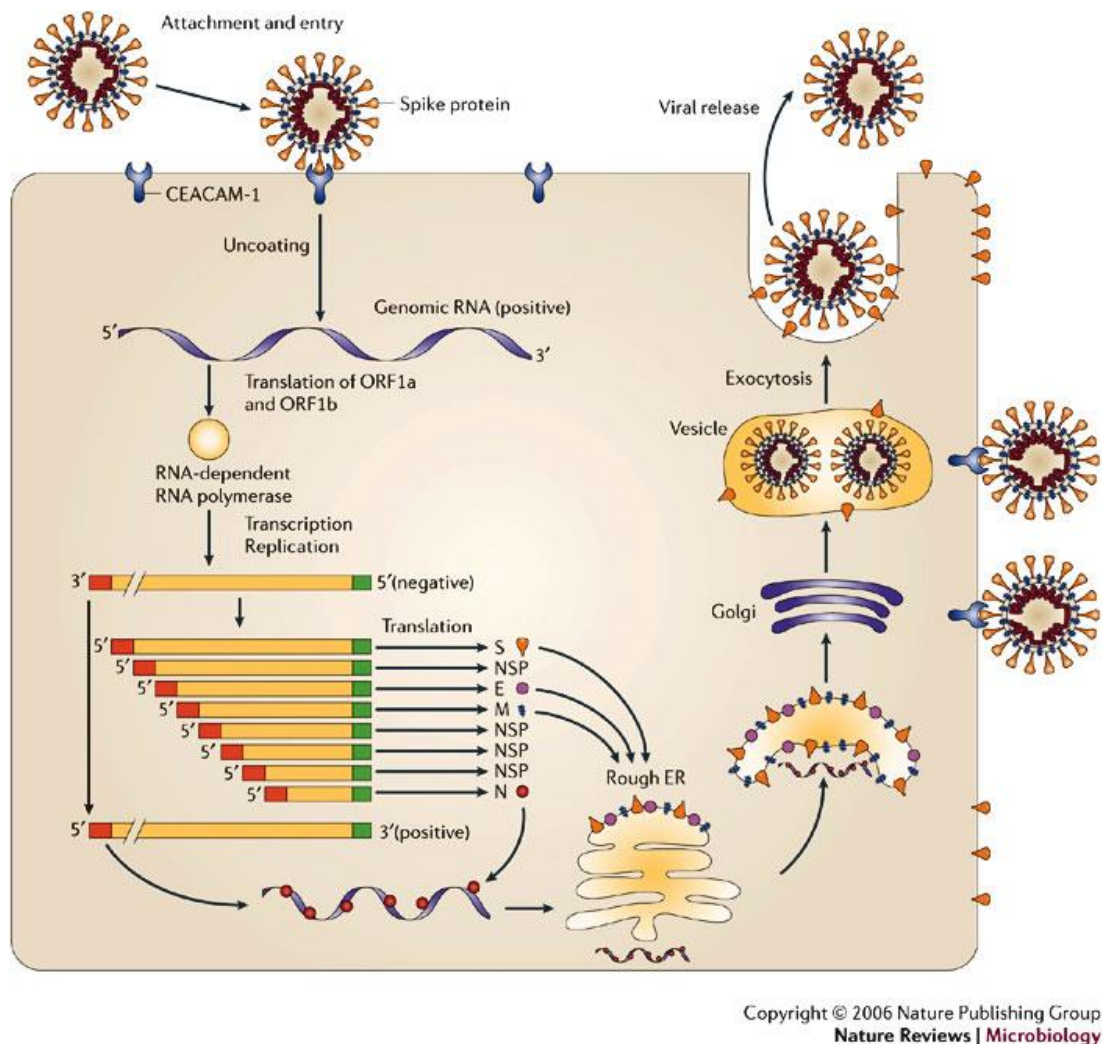


Figure 8: Life cycle of Mouse Hepatitis Virus
(Bergmann, Lane et al. 2006)

Infection starts as the spike protein first binds to receptors (MHVR-1) on the cell surface (Lai and Cavanagh 1997). The viral envelope then fuses with the cell membrane and the nucleocapsid is introduced into the cytoplasm of the host cell (Krzystyniak and Dupuy 1984). The first step of replication involves the translation of the genomic RNA to produce a viral RNA-dependent RNA polymerase, which can then copy the genetic material (Adami, Pooley et al. 1995). By discontinuous leader-primed transcription the seven mRNAs are then generated. Virus assembly takes place when the nucleoprotein binds to the genomic RNA to form the nucleocapsid, which in turn associates with aggregates of M proteins in the Golgi membranes. While the virus buds into the lumen of the Golgi apparatus host cell proteins in the lipid bilayer are replaced by virus

glycoprotein this. The virus is then released from the host cell by exocytosis or by cytolysis (Homberger 1997).

Several MHV strains can be recognised immunologically and by their tissue tropism, enterotropic and polytropic (Homberger, Zhang et al. 1998). Primary infection causes respiratory disease, hepatitis and enteritis. Enterotropic MHV strains are usually restricted to the intestine, with little dissemination to other organs (Casebolt, Qian et al. 1997) and are considered to be the most common form of natural infection (Barthold, Beck et al. 1993). As a result, virus is readily shed in the faeces. Infections can cause lesions in the intestine, variable levels of epithelial cell lysis and wasting away of villi in the terminal small intestine (Percy 2007).

Despite the clear distinction in pathology, nobody yet has found a way to differentiate between enterotropic and polytropic MHV strains *in vitro*. Comparison of the genome of enterotropic and polytropic MHV has not revealed a common distinguishing denominator. Most likely the tissue tropism determinant is located on one of the structural proteins, and is influencing the virions' ability to interact with the target cell (Homberger 1997).

Since some forms of MHV can cause serious disease and even mild infections have the potential to interfere with the collection of robust scientific data, most mouse breeding establishments will attempt to exclude it by having stringent constraints on the import of animals from other sources. The continued absence of the organism is then confirmed by regular screening, normally as part of tests for a range of pathogens.

1.2.4 Previous Studies

In the mid-20th century, MHV was studied as a model virus infecting mammalian systems (reviewed in (Weiss and Leibowitz 2011)). A number of strains were identified with different infectivity and virulence and attempts were made to understand the molecular bases of these properties in cell cultures and in animals.

Although the proteins on the virus surface (and their encoding RNA sequences) were found to be quite variable, more highly conserved genes were identified and targeted for the development of tests that would react against all strains of MHV.

The N protein (mRNA 7), situated towards the 3'end of the MHV viral genome, is the major structural component of virions and associates with genomic RNA to form a long,

flexible, helical nucleocapsid. This then interacts with the M protein, leading to the formation of mature virus particles. The N protein may play additional roles in viral transcription and translation, and/or replication (Ning, Lakatoo et al. 2003).

In the early 1990s, a Japanese group (Kunita, Terada et al. 1992) showed that *N* genes of MHV strains MHV-A59 and MHV-JHM possessed more than 92% nucleotide sequence homology and all sub-genomic mRNAs in infected cells shared the *N* gene sequence, making this a likely target for a PCR assay that would commonly be applicable to all the MHV strains with sufficient specificity and sensitivity. They compared the *N* genes of five strains of MHV and chose the primer sites for detecting MHV from the region of complete sequence conservation near the 3' end of the *N* gene. (Yamada, Yabe et al. 1998) also targeted the *N* gene to diagnose MHV using Nested PCR (figure 9). Primer location and sequences were based on the published MHV-JHMV mRNA 7 sequence (Skinner and Siddell 1983).

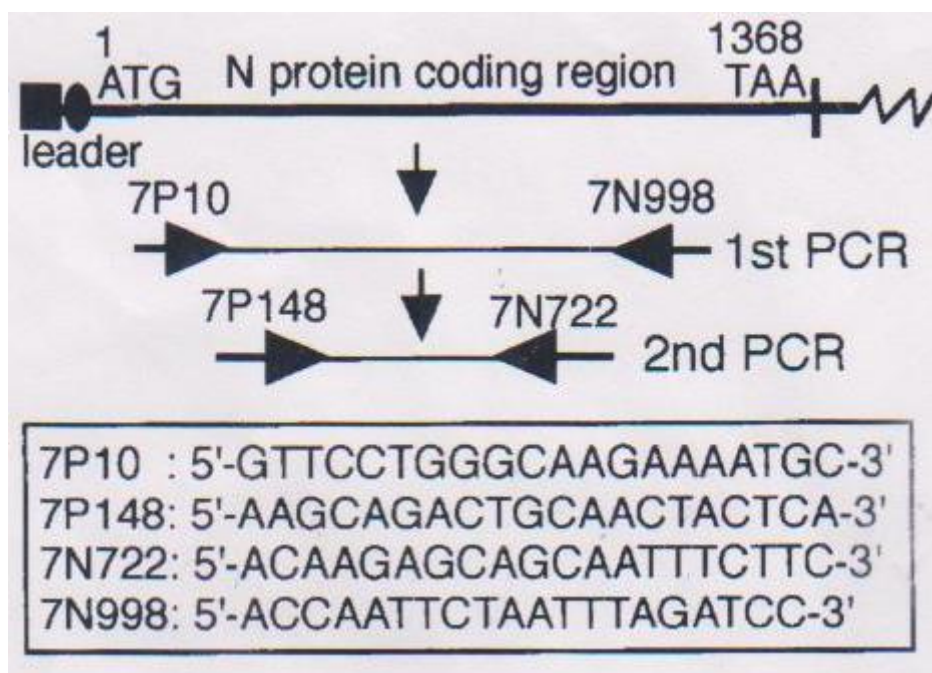


Figure 9: Primer location in the MHV mRNA 7 (N gene)

Primers used by (Yamada, Yabe et al. 1998) to carry out Nested PCR targeting the *N* gene

(Yamada, Yabe et al. 1998)

Other areas that were targeted were the polymerase gene (Casebolt, Qian et al. 1997) and membrane protein M (F R Homberger 1991). These are also highly conserved

regions of the viral genome. They exhibit high sequence homology among different MHV strains (F R Homberger 1991).

Previous sequencing by a commercial organisation of a PCR fragment from the N gene region of the genome of the virus circulating in the MSRU revealed a very close similarity with the A59 strain of MHV. (Figure 10)

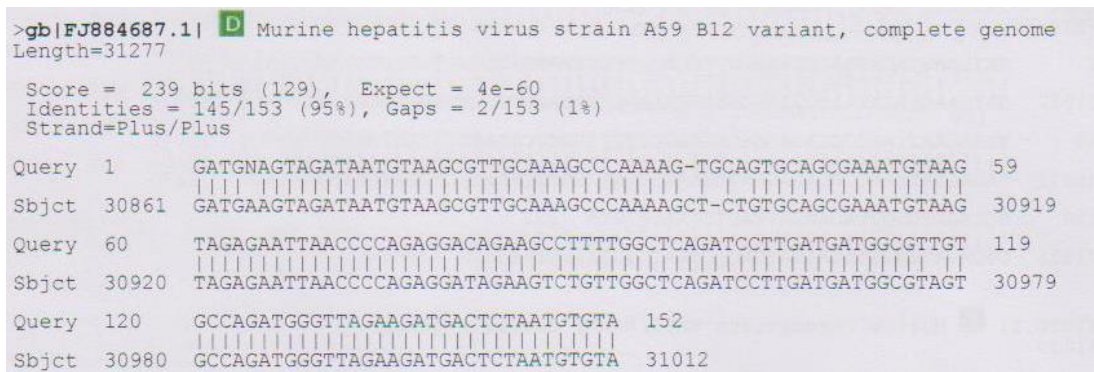


Figure 10: Comparison of N gene region of coronavirus circulating in the Medical School Resource Unit, Ninewells Hospital

The sequencing, done by a commercial company shows a 94% homology to MHV A59

Query = Sample from MRSU

Sbjct = Part of N gene of MHV A59

Even closer similarity with the N gene of a related virus, Puffinosis, a disease of manx shearwaters was in fact observed, though this was only as a result of nucleotide identities at one or two additional positions. As this is also a coronavirus, which is related to MHV (Nuttall and Harrap 1982) this could explain the close similarity.

It was decided that this study would also target the N gene as a site for amplification. Initially, the primers that were used were taken from a paper describing the use of PCR in the detection of MHV in an outbreak in a colony of immunodeficient mice (Yamada, Yabe et al. 1998). It was also initially thought that a second round of PCR would be required to be performed using another set of primers which targets a region within the first region (nested PCR). Nested PCR increases sensitivity, specificity and reduces amplification of unexpected primer sites. However, most of these studies were carried out in the 80's and 90's when PCR was in its infancy. With the advancement of new technology nested PCR might be too sensitive to even minute cross-contamination of the samples.

Viral RNA was extracted from faecal samples as these are easy to obtain non-invasively. This method was used by (Casebolt, Qian et al. 1997), (Yamada, Yabe et al. 1998) and

(Oyanagi, Kato et al. 2004). Some researchers used tissue samples (Wang, Campbell et al. 1999). Tissue samples of 94 mice were tested from suspected MHV infected non-nude mice, but positive results were only obtained in 8% of colon samples and 5% of a mixed sample of colon and liver. No positive results were obtained from faecal samples. When a nude mice was placed in the same cages with other non-nude mice or placed in cages with used dirty bedding, increased positivity was obtained, 10 out of 12 colon samples were positive (83%), and only 5 out of 10 faecal samples were positive (50%), however, the animal is required to be killed to collect the tissues. Choosing the correct time of testing is also an important consideration. Most laboratories agreed that the virus was rapidly cleared by immunocompetent mice and MHV genome may only be detectable in the period 3-21 days after inoculation.

1.3 Aims and Objectives

While the MHV strain circulating in the MSRU is not causing serious concern with respect to either animal welfare or the validity of current research projects, it is understandably almost impossible for researchers to share their animals with other laboratories. Certain types of research not currently undertaken in that unit, for example studies of the immune system, might be more seriously affected by the presence of the organism.

While the balance is still not in favour of a complete cull, fumigation and re-stocking (see above), a firewall strategy might still be worth attempting if much more were known about the life-cycle of the specific strain, how this might be affected by the different mouse strains in use and if a robust, inexpensive and rapid test for the presence of the virus itself were available. While PCR tests are now available from commercial suppliers, samples would still have to be collected, sent away, analysed and reported before actions could be taken on the basis of the data set. It might also not be affordable to test the number of samples necessary to be sure that the virus had indeed “burned out” before allowing the resumption of breeding.

The aim of this research project was to develop and validate a fast, easy and reliable method of testing for MHV, using an in-house PCR-based test. Furthermore, it was intended to use this test to begin to understand the life-cycle of the virus in the MSRU in order to evaluate the likely success or otherwise of a firewall strategy for its elimination.

2 Materials and Methods

2.1 Animals

C57BL/6J mice were obtained from Charles River Laboratories (UK) Ltd., at their “SPF/VAF” microbiological status. The supplier declared that the animals showed no evidence of previous exposure to MHV, among other organisms. The Charles River colony is periodically refreshed with animals from the Jackson Laboratories (Bar Harbor, USA), the “home” of the definitive C57BL/6J line. In some pilot experiments, samples were taken from other animals in the MSRU and other resource units in the University, but these too were on the same genetic background.

Five female and five male mice were received from Charles River at 6-8 weeks of age and housed in these two sex-matched groups. After the first phase of sample collection had been completed, three permanent mating pairs were set up. All animals were housed in conventional grid-top cages in a room in the facility that also accommodated several resident mouse colonies and were cared for by standard husbandry methods. A standard aspen sawdust substrate was used and RM1 diet (SDS Laboratories) and clean tap-water were provided ad libitum. Cages were changed weekly, with the cage bases being washed at high temperature but not autoclaved.

As the faecal pellet collection would not cause any significant pain, suffering, distress or lasting harm, and otherwise the animals received entirely standard care, the project did not fall under the authority of the Animals (Scientific Procedures) Act 1986. There were no untoward welfare issues throughout the project. At its end, all the animals were killed, with blood samples being collected post mortem from some of them for serological analysis (see below).

2.2 Faecal Collection

Mice were placed in a clean beaker until a sufficient number of faecal pellets were excreted. Pellets were then placed in a 2.0ml Eppendorf tube using clean tweezers and immediately put on ice. They were then either stored at -80°C or processed for RNA extraction immediately.

2.3 RNA Extraction – Qiagen Viral RNA Mini Kit (52904)

Buffers AVL, AW1 and AW2 were provided by the manufacturer of the kit.

Faecal samples were suspended in 1.5ml of 0.89% NaCl, scissor minced and centrifuged at 4000 x *g* using Eppendorf Centrifuge 5415R for 20 minutes. Supernatant was then filtered using 0.22µm filter (Sarstedt 83.1826-001), 140µl of each filtered sample were added to 560µl of prepared Buffer AVL containing carrier RNA in a 1.5ml micro-centrifuge tube and mixed by pulse-vortexing for 15 seconds. The samples were then incubated at room temperature for 10 minutes, then briefly centrifuged to remove drops from the inside of the lid. 100% Ethanol (VWR 20821.330) was added (560µl) to each sample and they were pulse-vortexed for 15 seconds. Each sample, buffer and ethanol solution (630µl) were then added to the Mini columns (in 2ml collection tubes) without wetting the rim. They were then centrifuged at 6000 x *g* for 1 minute. The Mini columns were then placed into clean 2ml collection tubes, and the tubes containing the filtrate were discarded. The samples were again centrifuged at 6000 x *g*. Buffer AW1 (500µl) was then added to each sample and centrifuged at 6000 x *g* for 1 minute. The Mini columns were then placed into clean 2ml collection tubes and the tubes containing the filtrate were discarded. Buffer AW2 (500µl) was added to the Mini columns and centrifuged at 20,000 x *g* for 3 minutes. The Mini columns were placed in clean 1.5ml micro-centrifuge tubes discarding the old collection tubes containing filtrate. The QIAamp Mini columns were carefully opened and 60µl of Buffer AVE equilibrated to room temperature was added to each of them. They were left at room temperature for 1 minute then centrifuged at 6000 x *g* for 1 minute. Samples were then stored at -20°C.

2.4 One-Step RT-PCR Kit

RT-PCR was carried out using Qiagen OneStep RT-PCR Kit (210210). Briefly, a master mix was prepared including the following components per sample:

DEPC treated H ₂ O	8.0µl
5x QIAGEN OnStep RT-PCR Buffer	5.0µl
dNTP mix	1.0µl
5x Q-Solution	5.0µl
Primer A	1.0µl
Primer B	1.0µl
QIAGEN OneStep RT-PCR Enzyme Mix	1.0µl
Total RNA	2.0µl

The master mix (23µl) was added to each tube along with the RNA sample (2µl) to give a total volume of 25µl and placed in VWR Thermocycler using the following program to amplify the products:

60°C – 30 minutes

95°C – 15 minutes

94°C - 1 minute

55°C – 1 minute

72°C – 1.5 minutes

35 Cycles

72°C - 10 minutes

4°C - Pause

2.5 Measurement of concentration and purity of RNA

Each RNA sample (2µl) was loaded onto a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific UK) and a measurement was taken. A modification of the Beer-Lambert equation was used to calculate sample concentrations:

$$C = (A * \epsilon) / b \text{ Absorbance} \times \text{Wavelength/Pathlength in cm}$$

where C is the nucleic acid concentration in ng/µl

A is the absorbance in AU (absorbance units)

ε is the wavelength-dependent extinction coefficient in ng-cm/µl

b is the pathlength in cm

Concentrations were calculated using the above described formula by the nanodrop software.

The absorption at 260 nm and the (260 nm/280 nm) absorption ratio was measured as an indication of the purity of the RNA. A ratio ~2.0 was taken as the optimal “pure” value.

2.6 RNA Extraction – Trizol Method

Total RNA was extracted using the Trizol method. Briefly, Trizol (Invitrogen 13547901) (1ml) was added to faecal samples (2, 5 and 8) which were then homogenised in short bursts using PT2500E Polytron homogeniser (Kinematica) and incubated at room temperature for 5 minutes. Chloroform (VWR 22706.326) (0.2ml) was added and the samples were shaken vigorously by hand for 15 seconds. Samples were incubated at room temperature for 3 minutes and then centrifuged at a speed of 12000 x g for 15 minutes at 4°C using Eppendorf Centrifuge 5415R. The aqueous phase was transferred to a fresh Eppendorf tube. RNA was precipitated by the addition of isopropanol (Fisher Scientific 1493415) (0.5ml); samples were incubated at room temperature for 10 minutes, and then centrifuged at 12000g for 10 minutes at 4°C. Supernatant was removed then 75%(v/v) Ethanol (VWR 20821.330) (1ml) was added and the sample was vortexed until the pellet dislodged from the tube. The sample was then centrifuged at 7500 x g for 5 minutes at 4°C, the ethanol carefully removed and the pellet air dried for 15 minutes before being dissolved in RNase-free water (Life Technologies AM9938) (1ml) and incubated at 65°C for 10 minutes. Samples were then stored at -20°C.

2.7 Reverse Transcription

Reverse transcription was carried out using Promega ImProm-II™ Reverse Transcription System (Promega UK Ltd A3800). Briefly, Total RNA (8µl), 10xRQ1 buffer (1µl) and RQ1 DNase (1µl) were added to an RNase-free sterile tube and the samples were incubated at 37°C for 30 minutes. RQ1 stop buffer (1µl) was added to each sample which was then incubated at 65°C for 10 minutes to inactivate the DNase. Samples were put on ice and DNase treated RNA (4µl) was then added to M3 primer (CAC ATT AGA GTC ATC TTC TA) (1µl) (the complement to the N gene of MHV) to make the cDNA and incubated at 70°C for 5 minutes. A master mix containing the following reagents was made up (all reagents came with Reverse Transcription System):

DEPC treated H ₂ O	7.3µl
ImPromII 5× reaction buffer	4.0µl
MgCl ₂	1.2µl
dNTP mix	1.0µl
Ribonuclease Inhibitor	0.5µl
ImProm II Reverse Transcriptase	1.0µl

Master mix (15µl) was added to each tube giving a total volume of 20µl. The samples were then incubated at:

25°C for 5 minutes

42°C for 1 hour

70°C for 15 minutes

Then stored at -20°C.

2.8 First strand cDNA Positive Control

To check that the synthesis of first-strand cDNA had worked using the ImProm-II Reverse Transcription System kit a test run was carried out using a positive control.

Kanamycin Positive Control RNA (1µg) was added (2µl of 1.2kb) to 1µl of Oligo(dT)₁₅ Primer (0.5µg/reaction) and 2µl of Nuclease-Free Water in a thin-walled dilution tube to give a final volume of 5µl. The tube was then placed into a preheated 70°C heat block for 5 minutes. It was then immediately chilled on ice for 5 minutes. The tube was then spun for 10 seconds in a micro-centrifuge. A reverse transcription reaction mix was made up including the following and added to the tube to make a final volume of 20µl:

Nuclease-Free H ₂ O	7.3µl
ImProm-II	4.0µl
MgCl ₂	1.2µl
dNTPs	1.0µl
Recombinant RNasin Ribonuclease Inhibitor	0.5µl
ImProm-II Reverse Transcriptase	1.0µl
Total Volume	15.0µl

The mixture was then incubated for the following times on a heat block:

Annealed	25°C – 5 minutes
Extended	42°C – 60 minutes
Inactivated	70°C – 15 minutes

The cDNA was then amplified by PCR by adding the above products to the following PCR mix and proceeded with thermal cycling on VWR Thermocycler:

Nuclease-free H ₂ O	16.0µl
10x Buffer	2.5µl
MgCl ₂	1.5µl
dNTPs	0.5µl
Forward Primer	1.0µl
Reverse Primer	1.0µl
Taq DNA polymerase	0.25µl
cDNA	1.0µl

Thermocycler Program:

94°C – 2minutes

94°C – 1minute

60°C – 1minutes

72°C – 2minutes

72°C – 5minutes

4°C - Pause

25 Cycles

2.9 Measurement of concentration of DNA

cDNA samples (2µl) were placed in the NanoDrop spectrophotometer and measurements taken as above. The ratio of absorbance at 260nm and 280nm was used to assess the purity, with a value of ~1.8 being taken as optimal for DNA.

2.10 Polymerase Chain Reaction

The following primers, which target regions within N protein of the MHV genome, were designed using Primer3 web version 4.0.0, (Koressaar T 2007; Untergrasser A 2012) and obtained from Eurofins MWG Operon. The N protein sequence was input into the program, which then produce a number of possible primers that could be used (figure 11)

ATGTCTTTTGTTCCTGGGCAAGAAAATGCCGGTGGCAGAAGCTCCTCTGTAAACCGCGCTGGTAATGG
 AATCCTCAAGAAGACCACTTGGGCTGACCAAAACCGAGCCTGGACCAAATAATCAAAATAGAGGCAGAA
 GGAATCAGCCAAAGCAGACTGCAACTACTCAACCCAACTCCGGGAGTGTGGTTCCCCATTACTCCTGG
 TTTTCTGGCATTACCCAGTTCCAAAAGGGAAAGGAGTTTCAGTTTGCAGAAGGACAAGGAGTGCTAT
 TGCCAATGGAATCCCGCTTCAGAGCAAAAGGGATATTGGTATAGACACAACCGCCGTTCTTTTAAAA
 CACCTGATGGGCAGCAGAAGCAATTACTGCCAGATGGTATTTTTACTATCTTGGCACAGGGCCCCAT
 GCTGGAGCCAGTTATGGAGACAGCATTGAAGGTGTCTTCTGGGTTGCAAACAGCCAAGCGGACACCAA
 TACCCGCTCTGATATTGTGCAAAGGGACCCAAGCAGTCATGAGGCTATTCCTACTAGGTTTGCGCCCG
 GCACGGTATTGCCTCAGGGCTTTTATGTTGAAGGCTCTGGAAGGTCTGCACCTGTAGCCGATCTGGT
 TCGCGGTCACAATCCCGTGGGCCAAATAATCGCGCTAGAAGCAGTTCCAACCAGCGCCAGCCTGCCTC
 TACTGTAAACCTGATATGGCCGAAGAAATTGCTGCTCTTGTGTTGGCTAAGCTCGGTAAAGATGCCG
 GCCAGCCCAAGCAAGTAACGAAGCAAGTGCCAAAGAAGTCAGGCAGAAAATTTTAAACAAGCCTCGC
 CAAAAGAGGACTCCAAACAAGCAGTGCCAGTGCAGCAGTGTTTTGGAAAGAGAGGCCCAATCAGAA
 TTTTGGAGGCTCTGAAATGTTAAACTTGGAACTAGTGATCCACAGTTCCCCATTCTTGCAGAGTTGG
 CTCCAACAGTTGGTGCCTTCTTCTTTGGATCTAAATTAGAATTGGTCAAAAAGAATTCTGGTGGTGTCT
 GATGAACCCACCAAAGATGTGTATGAGCTGCAATATTCAGGTGCAGTTAGATTTGATAGTACTCTACC
 TGGTTTTGAGACTATCATGAAAGTGTGAAATGAGAATTTGAATGCCTACCAGAAGGATGGTGGTGCAG
 ATGTGGTGAGCCCAAAGCCCCAAAGAAAAGGGCGTAGACAGGCTCAGGAAAAGAAAGATGAAGTAGAT
 AATGTAAGCGTTGCAAAGCCCAAAAGCTCTGTGCAGCGAAATGTAAGTAGAGAATTAACCCAGAGGA
 TAGAAGTCTGTTGGCTCAGATCCTTGATGATGGCGTAGTCCAGATGGGTTAGAAGATGACTCTAATG
 TGTAAT

Figure 11: Sequence of N gene including possible target primer sequences for amplification

Primers Left 1 forward and Right 1 reverse Primers Left 2 forward and Right 2 reverse
 Primers Left 3 forward and Right 3 reverse Primer left 4 forward

Table 4: New primers obtained from Primer3 web version 4.0.0 (Koressaar T 2007; Untergrasser A 2012) as the original primers used had high hairpin stability and unsuitable melting temperatures.

Primer	Start	Length	Melting Temp	Sequence
Left 1	2	21	58.97	TGTCTTTTGTTCCTGGGCAAG
Left2	248	20	57.74	TTGCAGAAGGACAAGGAGTG
Left3	754	20	58.77	CCCAAGCAAGTAACGAAGCA
Left4	1233	19	59.07	CGTTGCAAAGCCCCAAAAGC
Right1	120	20	58.34	ATTATTTGGTCCACGCTCGG
Right2 (unmodified)	625	20	60.04	ATTGTGACCGCGAACCAGAT
Right2 (modified)	625	18	58.03	ATTGTGACCGCGAACCAG
Right3 (unmodified)	1141	23	58.82	TCTGGTAGGCATTCAAATTCT CA
Right3 (modified)	1141	22	56.14	TCTGGTAGGCATTCAAATTCT C

The Left 1 and Right 3 primers were used as they targeted a large area at either end of the N gene and amplify a 1139bp DNA fragment, whereas the Left 2 and Right 2 pair targeted a smaller region in the middle of the N gene and amplify a 377bp DNA fragment. The Right 3 and Right 2 primers were modified to contain a C or G at the 3' in order to clamp the primer and prevent "breathing" of ends, with the expectation that this would increase priming efficiency. DNA "breathing" occurs when ends do not stay annealed but fray or split apart (Lorenz 2012). For the Right 3 primer, this was achieved by omitting the 3'-terminal A and for Right 2 primer, both the 3'-terminal A and T were omitted. The modified primers were used in this project.

Each sample (1µl) was added to a Master Mix of H₂O (16.25µl), 10x Dream Taq Buffer (Thermo Scientific EP0702) (2.5µl), dNTPs (Invitrogen 1214460) (0.5µl), BSA (Sigma A9418-100G) (2.5µl) forward and reverse primers (1µl) and Dream Taq (Thermo Scientific EP0702) DNA Polymerase (0.25µl), giving a total volume of 25µl. Each 25µl

PCR reaction therefore included: 10x Dream Taq Buffer (20mM MgCl₂), 0.2mM dNTPs, 400ng/μl BSA, 4μM primers and 50U/ml Dream Taq.

Amplification was performed on a VWR Thermocycler with an initial denaturation of 94°C for 3 minutes then 35 cycles of 30 seconds denaturation at 94°C, annealing at 54°C for Left 1 and Right 3, and 56°C for Left and Right 2, for 30 seconds, then primer extension at 72°C for 1 minute. The extension of the last cycle was continued for 10 minutes at 72°C.

2.11 Agarose gel electrophoresis

The resulting amplified DNA product was detected by electrophoresis run on a 1.5% agarose gel. UltraPure™ Agarose (1.5gm) (Invitrogen 16500-500) and 100ml 1× TE Buffer (100ml) were heated for 1 minute and then 3μl Sybr® Safe DNA gel stain (Invitrogen S33102) was added before casting the gel. 2.5μl dye was then added to each 25μl sample, 10μl of which were then pipetted into a gel slot. A 1kb GeneRuler DNA ladder (Thermo Scientific #SM0311) was also added to the gel to confirm the correct band sizes. Electrophoresis was for 1 hour at 100volts, after which the nucleic acids were visualised by UV illumination using a Syngene U:Genius 3 illuminator.

2.12 Taqman Analysis

Newly extracted and Reverse Transcribed samples were diluted to 1:80 i.e. 2μl of each cDNA sample was added to 158μl of ddH₂O to give a total volume of 160μl. A master mix of the following was made up in triplicate for each sample:

18s Primer and Probe (1256325 Applied Biosystems)	1μl
---	-----

Hmox1 gene Primer and Probe (1170383 Applied Biosystems)	1μl
--	-----

Taqman Gene expression Master Mix (4369016 Applied Biosystems) 10μl

12μl of master mix was added in triplicate to a 96well plate plus 8μl of diluted cDNA for each sample.

This was then run on Taqman Real Time PCR system using 7500 system software.

2.13 “Positive control” DNA

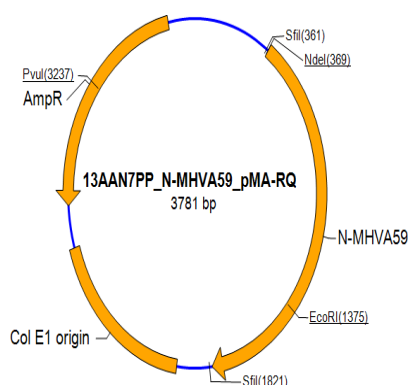
A synthetic cDNA of the N gene, based on the database sequence for the A59 strain of MHV was obtained from Life Technologies to be used as a positive control (Figure 12). It was assembled from a number of overlapping synthetic oligonucleotides and inserted into a plasmid for cloning and extraction.

Plasmid DNA Description:

The synthetic gene N-MHVA59 was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into pMA-RQ (amp^R) using SfiI and SfiI cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%. See the accompanying data sheets for sequences and final ABI trace files as well as the assembled sequences electronically on disk. 5 µg of the plasmid preparation were lyophilized for shipping.



Plasmid Map:



Quality Assurance Documentation: 13AAN7PP

Ref. No.: 1340439

Designation: E.coli K12 (dam⁺ dcm⁺ tonA⁻ rec⁻)

Gene name: N-MHVA59

Gene size: 1440 bp

Vector backbone: pMA-RQ (amp^R)

Cloning sites: SfiI / SfiI

Quantity: ~5 µg Plasmid DNA

Note: Please dissolve lyophilized DNA in 50 µl distilled water or 10 mM Tris-HCl (pH 8.0). We recommend sequence verification after each transformation step.

Date: 22 May 2013

Anja Philipps

Quality control

GeneArt AG www.lifetechnologies.com GeneArtSupport@lifetech.com

Figure 12: Description of Synthetic DNA obtained from Life Technologies

2.14 DNA transformation

The plasmid DNA was transformed into 10-beta E. coli bacteria (New England BioLabs C3019H).

Briefly, 1 µl of plasmid DNA was added to 50 µl competent cell mixture (10-Beta). The tube was flicked 4-5 times to mix the cells and DNA. The mixture was then placed on ice for 30 minutes. It was then placed in an Eppendorf Thermomixer Compact at a temperature of 42°C for 30 seconds in order to open the cells by heatshock. The sample was then placed on ice for 2 minutes in order to close the cells. 950 µl of SOC medium was then added and it was placed in a shaking incubator at 37°C for 60

minutes. Cell mixture (50-100µl) was then spread on LB agar plates containing 100µg/ml of LB-ampicillin and incubated overnight at 37°C in an incubator. Two colonies were then grown in 5ml of LB-ampicillin in a 37°C incubator with 250rpm shaking for 8 hours. Then 200µl of the 5ml culture was transferred to 100ml LB-ampicillin and incubated overnight at 37°C with 250rpm shaking.

The DNA was then extracted from the 100ml overnight culture and purified using a Qiagen Midi Kit (13343) as per the manufacturer's instructions.

2.15 “Negative control” DNA

For some experiments, faecal samples were collected from C57BL/6J mice held in another resource unit in the University, in which MHV has never been detected. These were shipped to the laboratory at ambient temperature and then processed immediately for RNA extraction.

Distilled water was also used as a negative control.

2.16 Serological testing

After animals were killed, a single spot of blood was blotted onto “OptiSpot” paper (supplied by IDEXX). After drying, the samples were stored at 4°C before being shipped to IDEXX Laboratories for MHV serology testing.

3 Results

3.1 Trizol method and RT-PCR Vs RNA mini kit and one-step PCR

The extraction and PCR were tried two different ways. The first way was using a “Mini Kit” designed for RNA extraction and then a one-tube process for reverse transcription and PCR amplification. The second was RNA extraction using the standard Trizol method, which would be followed by reverse transcription and PCR.

Gross RNA yields are compared in figures 13 and 14. The Trizol method proved to give a higher yield, however, the differences in yield is likely representative of extracting RNA from whole pellets (Trizol) vs. filtered supernatants (mini kit). In addition, no positive results were obtained after three attempts with the one-tube PCR reaction after use of the Mini Kit. The one-tube PCR reaction was not tried with the Trizol method and vice versa. Trizol method of RNA extraction, followed by separate reverse transcription and PCR steps was adopted as the standard procedure in further work as this gave positive results after the first attempt.

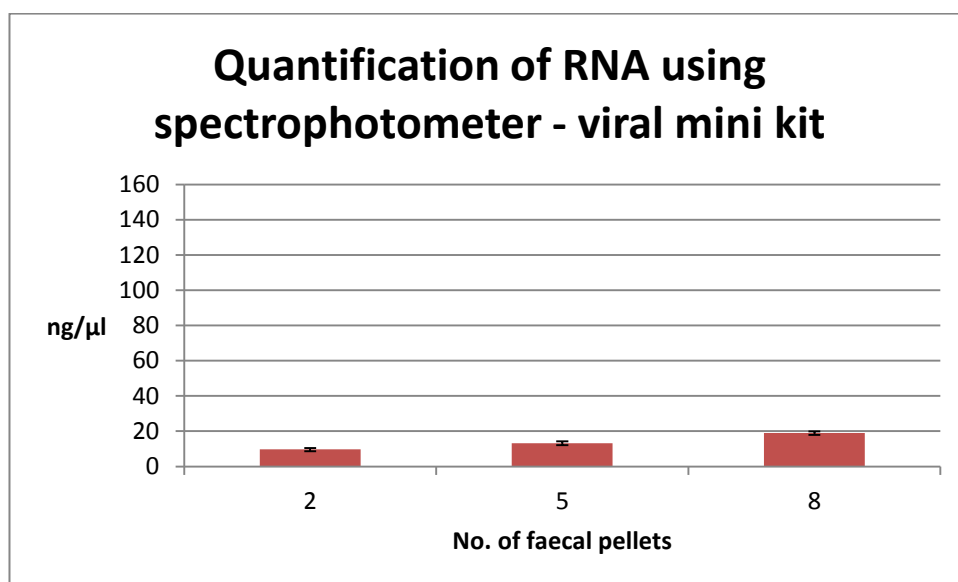


Figure 13: RNA yield using viral mini kit. Amount of RNA (ng/μl) in samples containing 2, 5 and 8 faecal pellets.

Data represents mean \pm standard deviation, N=3 determinations

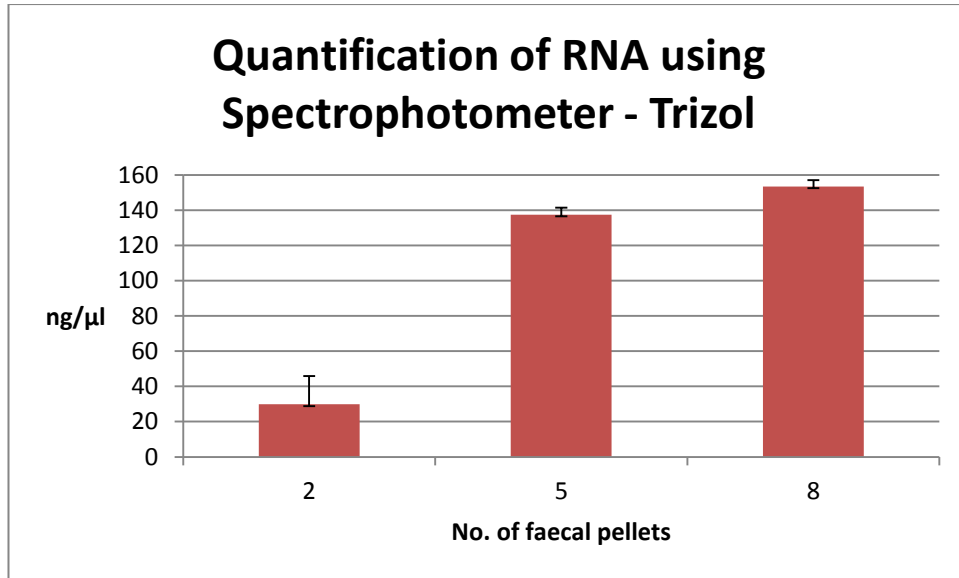


Figure 13: RNA yield using Trizol extraction. Amount of RNA (ng/μl) in samples containing 2, 5 and 8 faecal pellets.

Data represents mean \pm standard deviation, N=3 determinations

3.2 RNA yield from faecal pellets

Fresh faecal samples were collected in different numbers from randomly chosen mice in the general population of the MSRU and RNA was extracted using the Trizol method. RNA yield versus number of pellets was compared. RNA yield increased in a linear fashion from one to three pellets, however, any further increase in pellet numbers did not increase RNA yield significantly (Figure 15), indicating a saturation of the extraction method at the quantities of reagents used. Therefore, three pellets were used in all future work, as this gave the maximum RNA yield within the linear range.

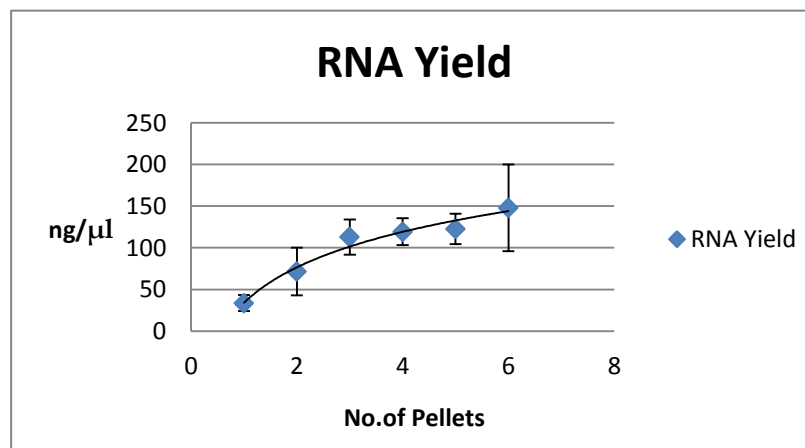


Figure 14: RNA yield of faecal pellets. Amount of ng of RNA per μl of sample for 1-6 faecal pellets.

Data represents mean \pm standard deviation, N=3 determinations

3.3 Tests for amplification

It was important to verify that the extracted RNA could indeed be reverse-transcribed into cDNA and that this could then yield a PCR amplification product. Two approaches were used, both depending on the likely presence in faecal material of cellular debris from the gastrointestinal tract.

3.3.1 Analysis of haem oxygenase-1 expression

Faecal samples were collected from mice that were involved in a study of haem oxygenase-1 (HO-1) expression - “Evidence that the capacity of nongenotoxic carcinogens to induce oxidative stress is subject to marked variability”(Henderson, Cameron et al.), and processed by the standard methods. A Taqman analysis was performed on samples from control animals and mice in which HO-1 expression was expected to be elevated (Figure 16). A significant increase in this signal was indeed detected.

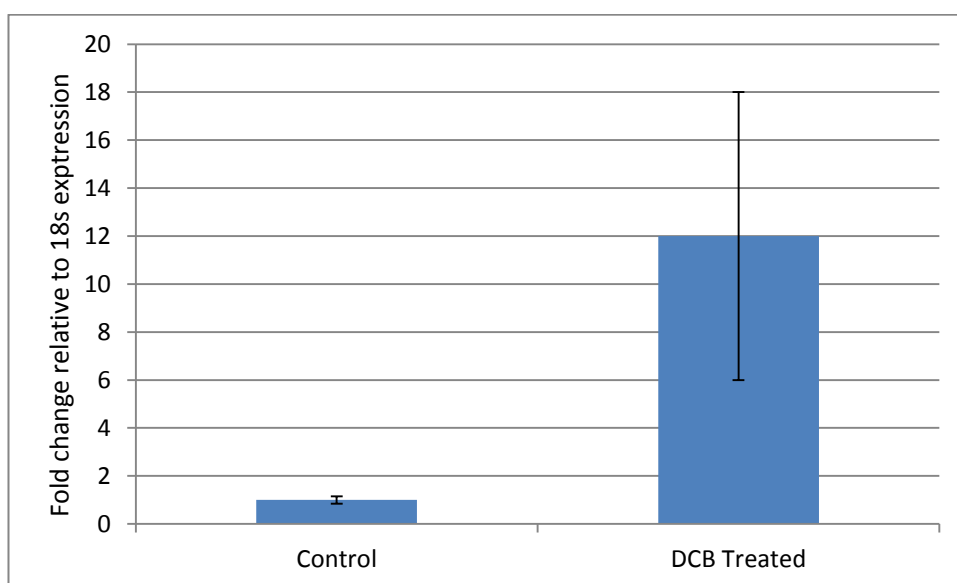


Figure 15: Taqman test was performed using HO-1. Control – Haemoxygenase-1 Dual Reporter (HOD) mouse treated with corn oil and Dichlorobenzene (DCB) treated HOD mouse.

3.3.2 Actin expression

Reverse transcription and PCR reactions were carried out, targeting the mRNA for β -Actin, a ubiquitous protein that is present in mammalian cells. A PCR product of the expected size was indeed obtained (Figure 17).

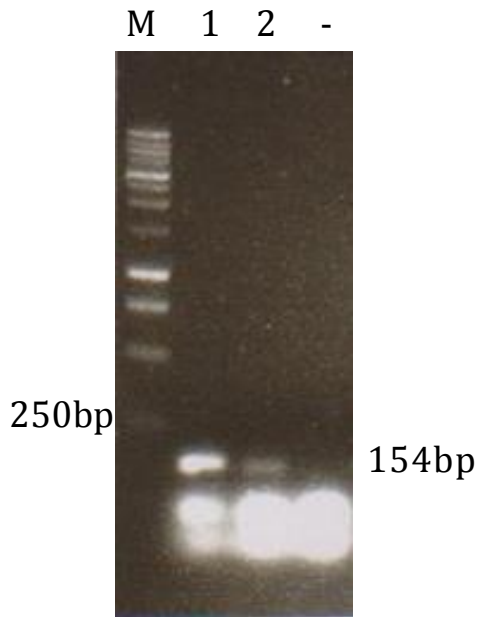


Figure 16: Results of RT-PCR to detect β -actin in a faecal sample. M – Ladder, 1 – Non-diluted cDNA, 2 – cDNA diluted 1:10, 3 – Negative Control (H_2O)

3.4 Synthetic Positive Control

Synthetic cDNA of N-MHVA59 gene was obtained from Life Technologies to be used as a positive control (Figure 12). After transformation the plasmid DNA stock was diluted to 50ng/ μ l, 5ng/ μ l, 0.5ng/ μ l, 0.05ng/ μ l and 0.005ng/ μ l and used as template DNA for PCR reactions using the primer pairs: P1forward and P3 reverse and P2 forward and reverse (modified primers). An example of the PCR products formed is shown in figures 18 and 19. These bands correspond to the predicted molecular weights for each primer pair.

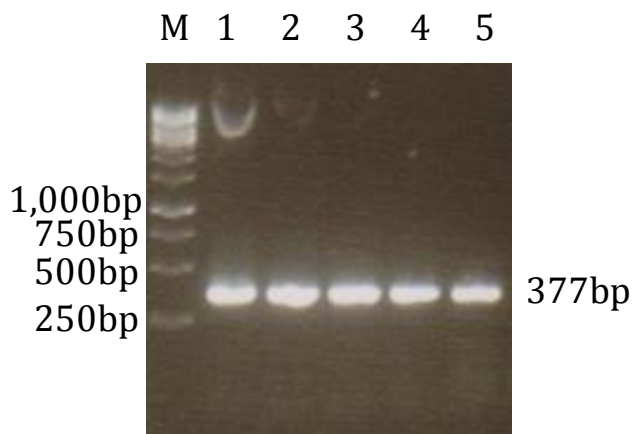


Figure 17: Agarose gel electrophoresis of PCR product of diluted plasmid DNA stock using primers P2 forward and reverse.

M = DNA marker ladder, 1-5 = diluted DNA 5ng/ μ l, 5ng/ μ l, 0.5ng/ μ l, 0.05ng/ μ l, 0.005ng/ μ l

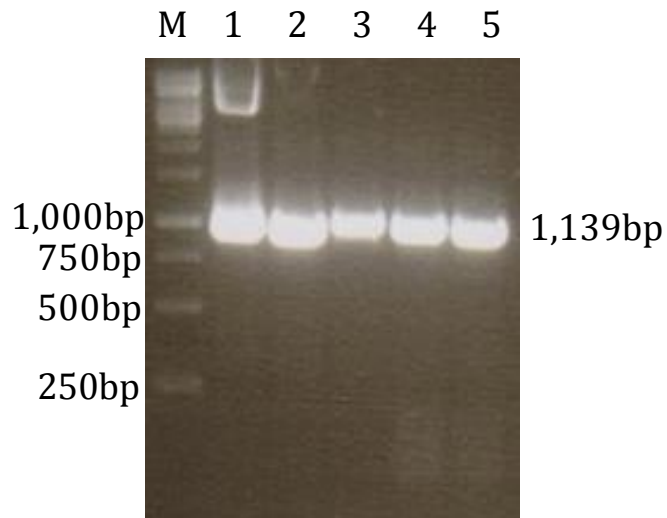


Figure 18: Agarose gel electrophoresis of PCR product of diluted plasmid DNA stock using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-5 = diluted DNA 5ng/μl, 5ng/μl, 0.5ng/μl, 0.05ng/μl, 0.005ng/μl

3.5 First-strand cDNA Positive Control

To check that the synthesis of first-strand cDNA had worked using the ImProm-II Reverse Transcription System kit a test run was carried out using a positive control (Figure 20). This positive control was included with the ImProm-II kit and the reaction was run as per the manufacturer's instructions (see sections 2.8 of materials and methods)

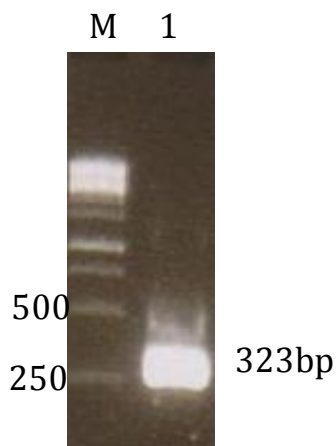


Figure 19: 1.2kb Kanamycin Positive Control
M= DNA marker ladder, 1=Positive Control

3.6 Amplification of MHV N-gene fragment from extracted RNA

Faecal samples were collected from 10 random weanling mice in various rooms within the general population of the animal unit. All animals were between 3-21 days after weaning, at a time when maternal passive immunity was likely to have disappeared. Figure 21 shows the results of one of the groups of mice samples were collected from, using primers Left 1 (P1) and Right 3 (P3). Three mice tested positive for MHV.

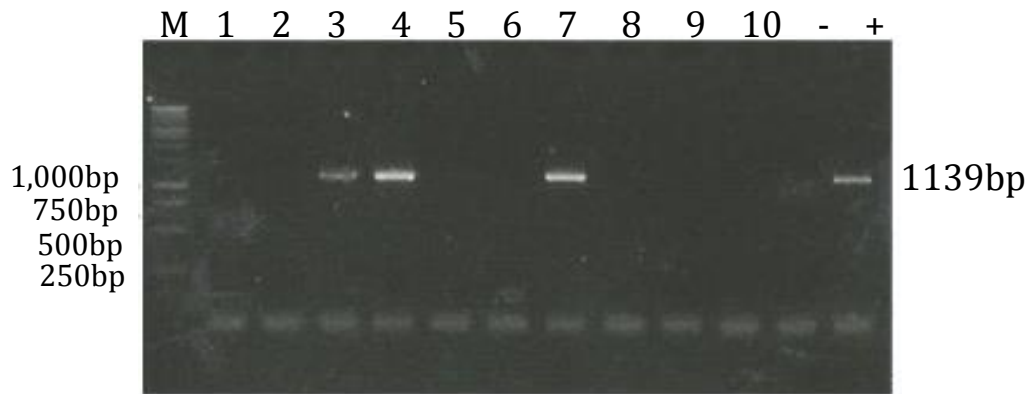


Figure 20: RT-PCR amplification of MHV N gene from fresh faecal samples

RNA was prepared from fresh faecal samples from 10 random weanling mice in MSRU, reverse transcribed and PCR for the MHV N gene with Primers P1 Forward and P3 Reverse carried out and run. Agarose gel electrophoresis was run as detailed in Materials and Methods.

M – DNA marker ladder. Lanes 1,2,5,6,8,9,10 – negative results. Lanes 3,4,7 – positive results with the correct band of 1139bp.

A PCR was then run again on the positive samples (3, 4, and 7) using Primer 2 forward and 2 reverse. These also tested positive for MHV (Figure 22).

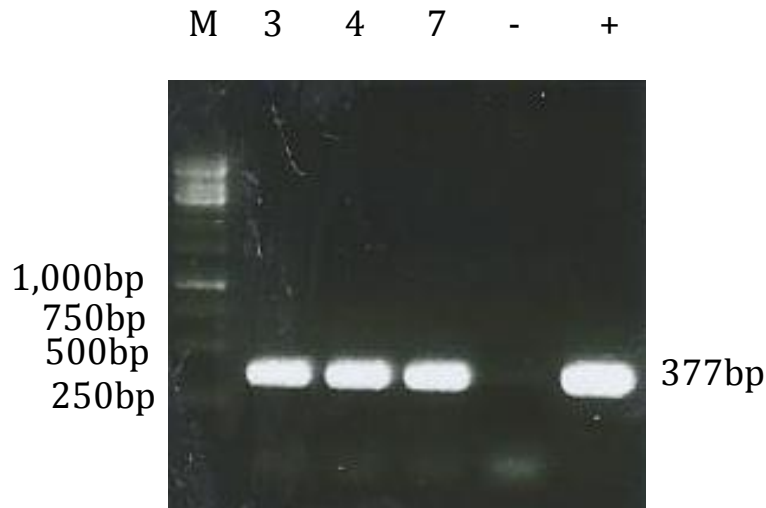


Figure 21: PCR amplification of MHV gene using primers P2 forward and P2 reverse L – DNA Marker Ladder. Lanes 3, 4, and 7 showing positive results with correct band of 377bp.

3.7 Sequencing of Positive Result

The PCR product of one of the positive results (sample 3 above) was sent for sequencing to the Medical School Core DNA Sequencing Facility analysis, using primer P1-fwd. Results are shown in figure 23, and demonstrate a sequence identity of 88% with the N-gene as recorded in the standard sequence databases. The PCR product was clearly highly related to the viral sequence (note that an exhaustive and repeated comparison was not performed, so sequencing errors cannot be ruled out as being responsible for being some or all of the differences).

N-Gene	132	CAGAAGGAATCAGCCAAAGCAGACTGCAACTACTCAACCCAACTCCGGGAGTGTGGTTC	191
MS1	86	CAGGAGAAACCAGCCAAAGCAKWCTGCAACTACTCAGTCCAACCCCGGGAGTGTGGTTC	145
N-Gene	192	CCATTACTCCTGGTTTCTTGGCATTACCCAGTTCCTAAAAGGGAAAGGAGTTTCAGTTTGC	251
MS1	146	CTATTACTCCTGGTTTTCGGGCATTACCCAATTCCAGAGGGGAAAGCAATTCCAGTTTGC	205
N-Gene	252	AGAAGGACAAGGAGTGCCATTGCCAATGGAATCCCGCTTCAGAGCAAAAGGGATATTG	311
MS1	206	AGAAGGACAAGGAGTGCCATTGCCAATGGAATCCAGCCACTGAGCAAAAGGGATATTG	265
N-Gene	312	GTATAGACACAACCGCGTTCCTTTTAAACACCTGATGGGCA-GCAGAAGCAATTACTGC	370
MS1	266	GTTTAGACACAACCGCGTTCCTTTTAAACACCTGATGGGCAAAACA-AAACAACCTTTTAC	324
N-Gene	371	CCAGATGGTATTTTTACTATCTTGGCACAGGGCCCCATGCTGGAGCCAGTTATGGAGACA	430
MS1	325	CCCGCTGGTATTTTTACTATCTCGGCACTGGACCCTATGCCTCTGCCGAATATGGCGATG	384
N-Gene	431	GCATTGAAGGTGTCTTCTGGGTG-CAAACAGCCAAGCGGACACCAATACCCGCT-CTGA	488
MS1	385	ACATCGAAGGAGTCTGTTGGGTCGCCAATAAG-CAGGCTGACATTAAGACC-GCTGCCGA	442
N-Gene	489	TATTGTCGAAAGGGACCCAAGCAGTCATGAGGCTATTCCTACTAGGTTTGCGCCCGGCAC	548
MS1	443	TATTGCTGAGAGGGACCCAAGCAGTCATGAGGCTATTCCTACTAGGTTTGCGCCCGGTAC	502

Figure 22 Comparison of partial sequence (380bp) of N gene of MHV genomic RNA from a positive mouse sample (MS1)

3.8 Serial dilution of positive sample

To assess the sensitivity of the RT-PCR, one of the positive mouse samples was serially diluted 1:2 with water. By the fourth dilution (Figure 24, lane 5) no product can be seen. This confirms RT-PCR should be sensitive enough to detect the virus over some range of concentrations in the samples. It does not, however, determine the limit of sensitivity of the assay.

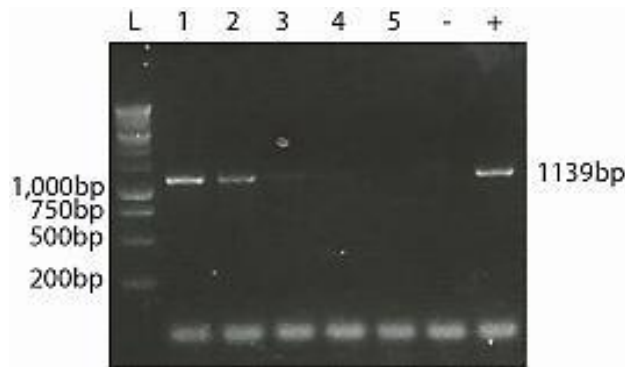


Figure 23: Serial dilution of a positive sample. L – DNA marker. Lane 1 – no dilution. Lanes 2 – 5: serial dilution 1:2 with H₂O

3.9 Nested PCR

It was first thought that a nested PCR would be required as this had been used in previous studies with good results. As the virus was being collected from faecal samples it was assumed that any virus extracted would be limited to very small amounts and a nested PCR is more sensitive. Primers Left 1 and Right 3 were used in the first round of PCR as this targeted a large area of the N gene, and primers Left 2 and right 2 were used in the second round of PCR as this targeted a smaller area within the large area. Initially results were good with the second round of PCR giving a positive result when the first round did not. However, after running a few of these PCRs there were signs of contamination, with many of the negative control (water) samples giving a positive result also. It was concluded that the likely efficiency of PCR would have been improved greatly since the first reports of its use in detecting MHV and that the added sensitivity of a nested approach might now be detecting an irreducible “background” (see below). Therefore, the further use of nested PCR was abandoned.

3.10 Contamination issues

At several points during the project, serious contamination issues were encountered. There was contamination caused by the plasmid positive control which was combated, by using this highly concentrated source of viral sequences as rarely as possible, and handling it in strict isolation in the fume hood. All master mix components were also aliquoted into single reaction amounts in order to reduce cross contamination. This worked well eliminating contamination. Figures 25 and 26 show examples of gels that show contamination.

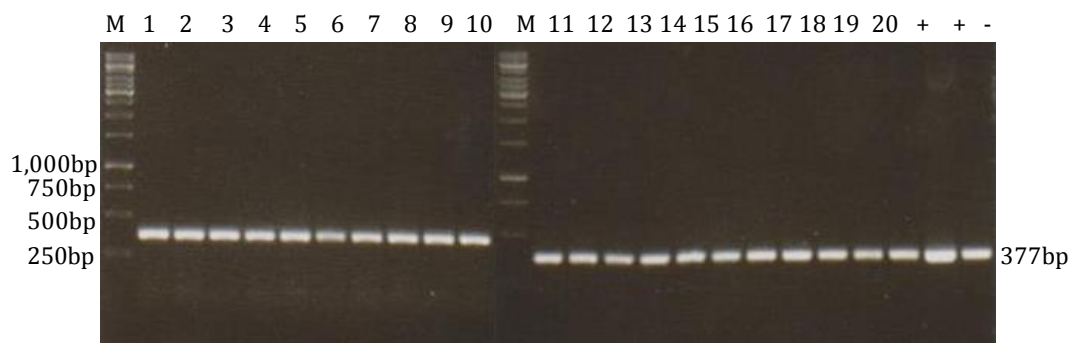


Figure 24: Example of contaminated samples of mouse 5 using primers P2 forward and reverse. All samples and negative control show contamination.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

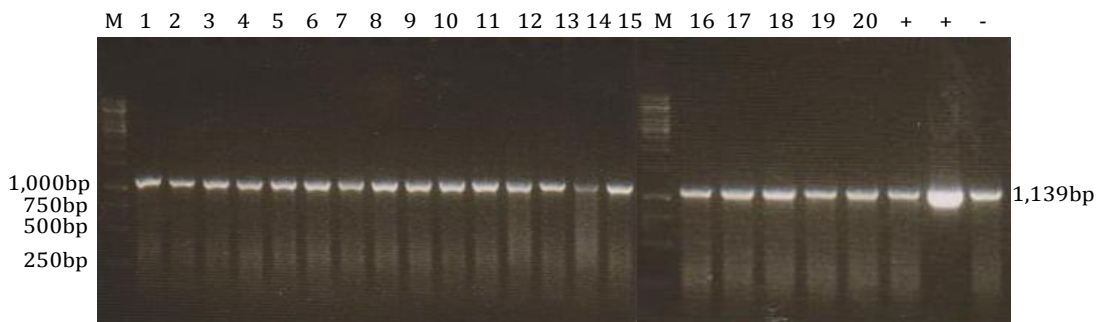


Figure 25: Example of contaminated samples of mouse 5 using primers P1 forward and P3 reverse. All samples and negative control show contamination.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

3.11 Optimisation of the PCRs

Gradient PCRs were performed on a known positive sample using both primer pairs (P1P3 and P2 forward and reverse) to determine the optimal annealing temperatures. For Primers P1 and P3 a range of between 48.6°C and 57.2°C was suitable, and for primers P2 forward and reverse a range of 48.6°C and 60.9°C gave the best results (figure 27 and 28). The number of cycles was also varied (30 and 35 cycles), however this made no difference to the outcomes of PCRs (figures 29 and 30).

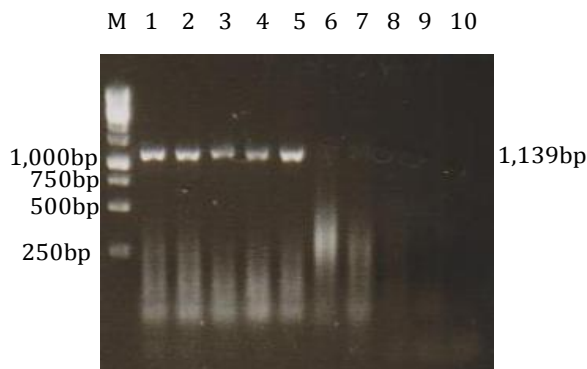


Figure 26: Gradient PCR performed on a known positive sample using Primers P1 and P3

M=DNA ladder, 1=48.6°C, 2=49.9°C, 3=51.2°C, 4=54.0°C, 5=57.2°C, 6=60.9°C, 7=64.0°, 8=66.5°C, 9=68.2°C 10=69.5°C

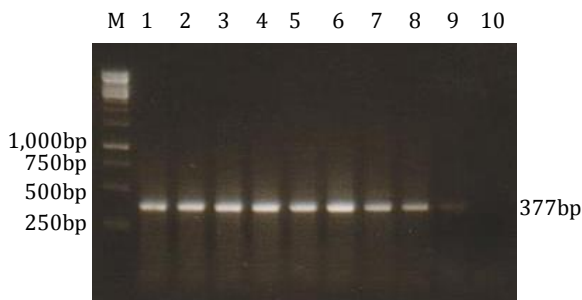


Figure 27: Gradient PCR performed on a known positive sample using Primers P2 forward and reverse

M=DNA ladder, 1=48.6°C, 2=49.9°C, 3=51.2°C, 4=54.0°C, 5=57.2°C, 6=60.9°C, 7=64.0°, 8=66.5°C, 9=68.2°C 10=69.5°C

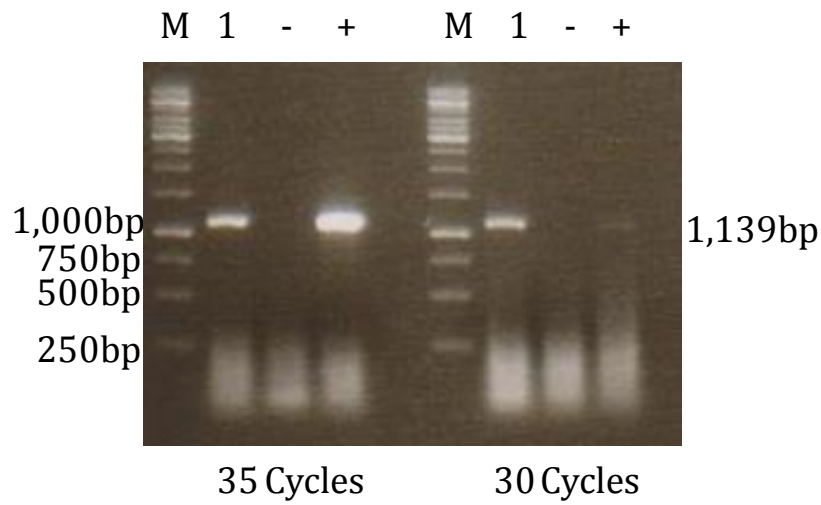


Figure 28: PCRs performed with Primers P1 and P3 using two different numbers of cycles

M = DNA ladder, 1 = Known positive sample, - = Negative control (H₂O), + = Plasmid positive control

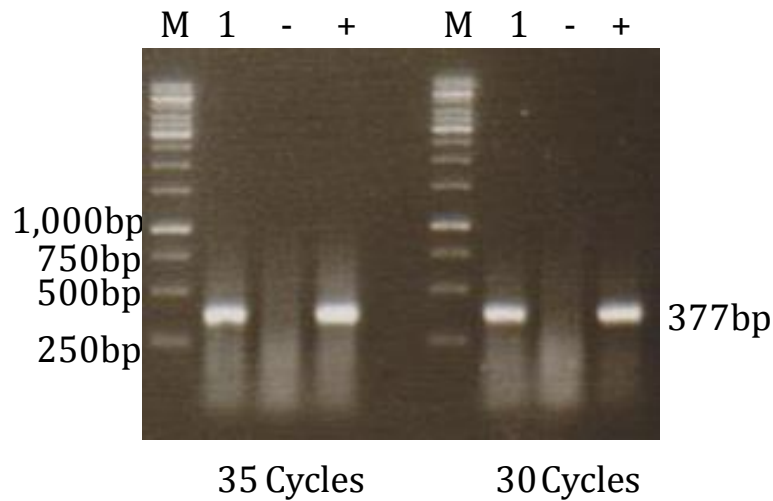


Figure 29: PCRs performed with Primers P2 forward and reverse using two different numbers of cycles

M = DNA ladder, 1 = Known positive sample, - = Negative control (H₂O), + = Plasmid positive control

3.12 MHV detection in animals housed in the MSRU, from their arrival

RT-PCRs were performed on samples that were collected daily for 20 days (excluding weekends) from 5 male and 5 female 6-8 week old mice that were obtained from Charles River Laboratories (UK) Ltd, to determine if they had contracted MHV and for how long. Faecal collection started the day after they arrived.

Mouse 1

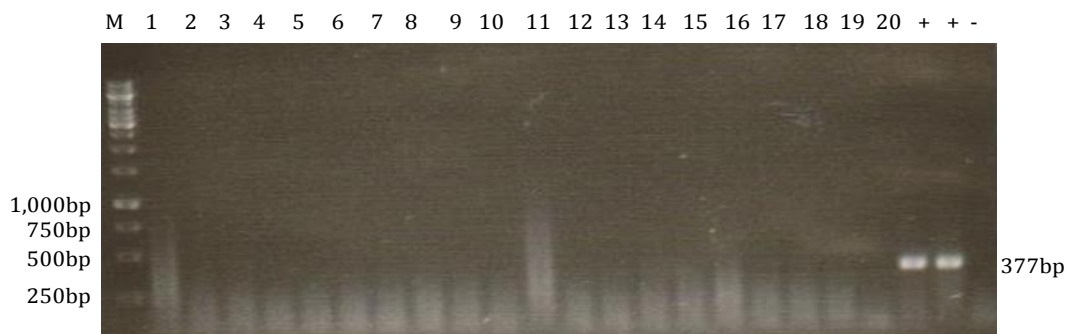


Figure 30: Agarose gel electrophoresis of total RNA and RT PCR product of mouse 1 (male) using Primers P2 forward and reverse.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known nMHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

No positive results



Figure 31: Agarose gel electrophoresis of total RNA and RT PCR product of mouse 1 (male) using Primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control.

No positive results

Mouse 2

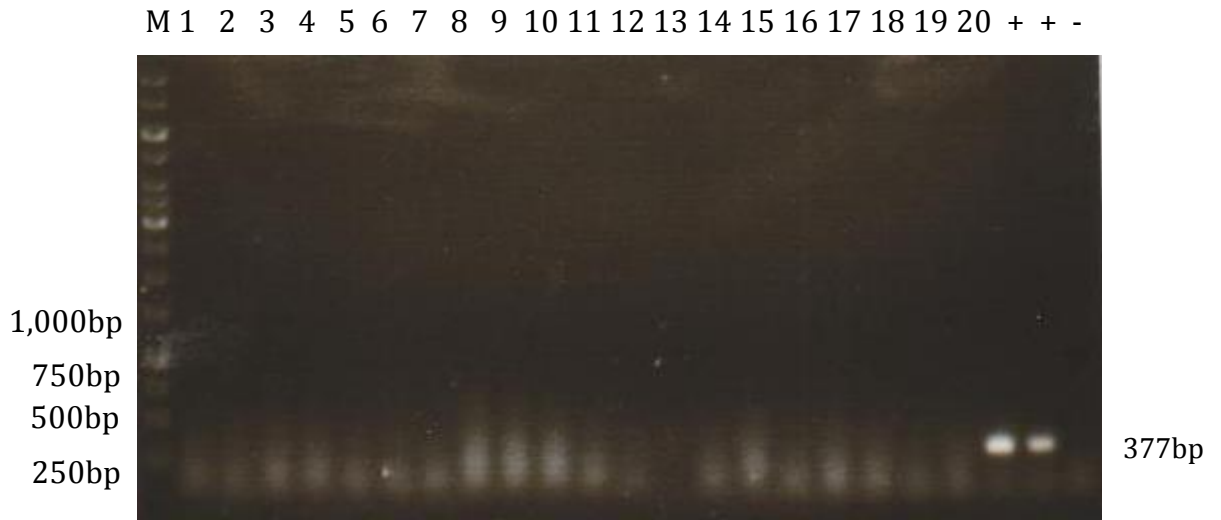


Figure 32: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 2 (male) using primers P2 forward and reverse.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

No positive results

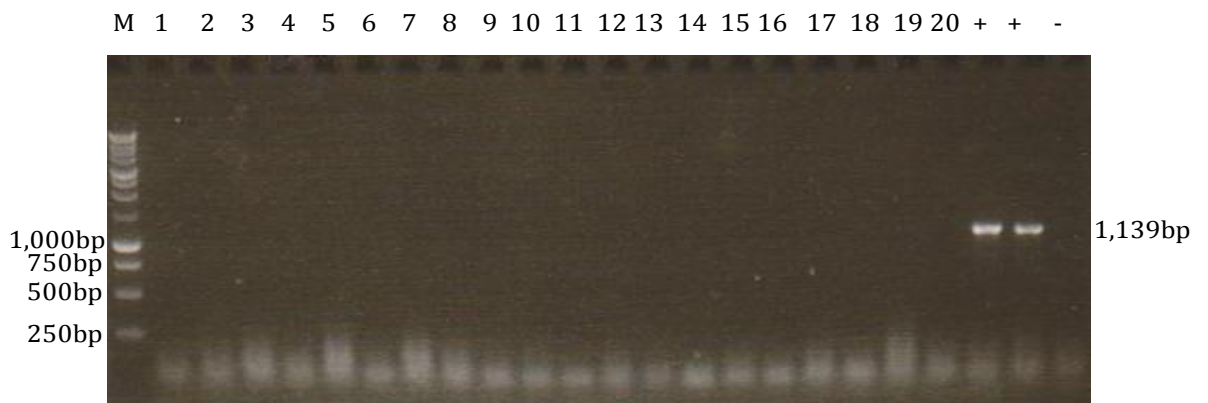


Figure 33: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 2 (male) using primers P1 forward and P3 reverse.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

No positive results

Mouse 3

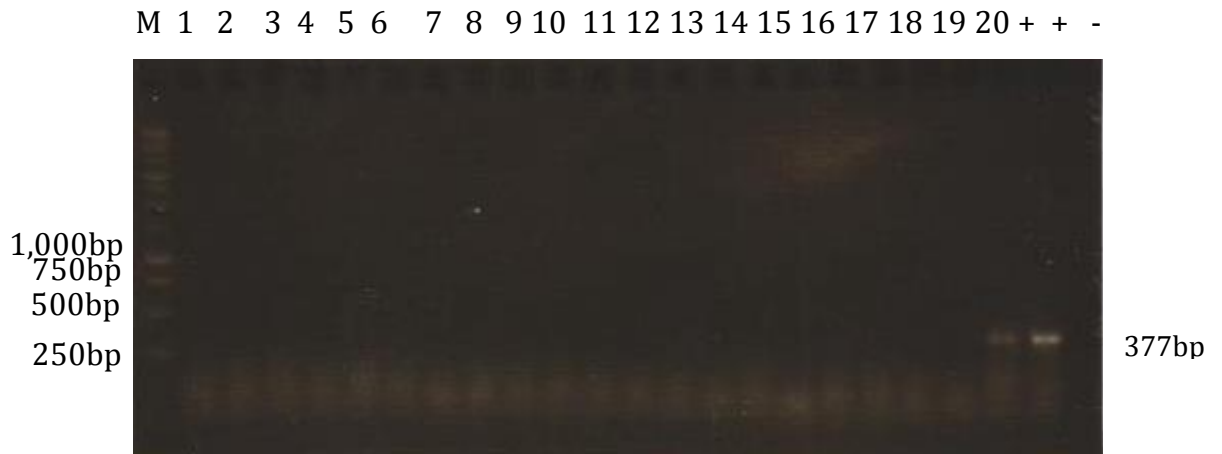


Figure 34: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 3 (male) using primers P2 forward and reverse.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

No positive results



Figure 35: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 3 (male) using primers P1 forward and P3 reverse.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

No positive results

Mouse 4



Figure 36: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 4 (male) using primers P2 forward and reverse.

M= DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MRSU, second + = synthetic positive control, - = negative control

No positive results



Figure 37: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 4 (male) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 5



Figure 38: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 5 (male) using primers P2 forward and reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results



Figure 39: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 5 (male) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 6



Figure 40: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 6 (female) using primers P2 forward and reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results



Figure 41: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 6 (female) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 7



Figure 42: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 7 (female) using primers P2 forward and reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

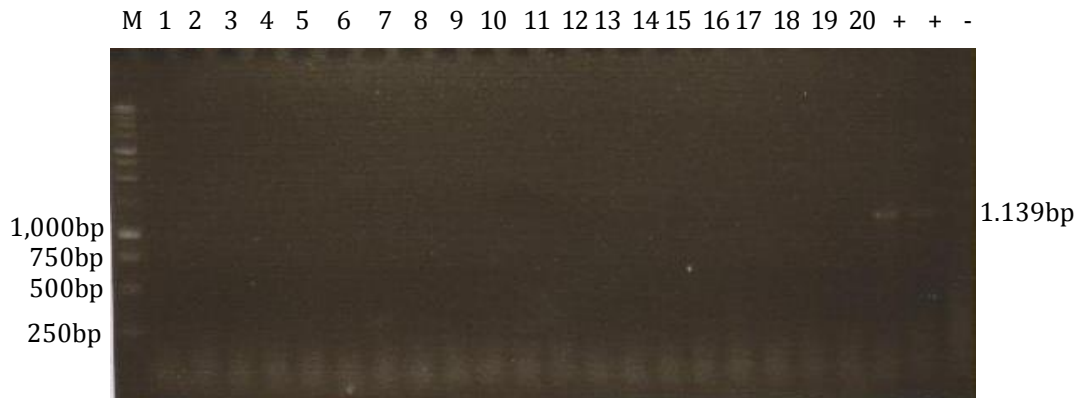


Figure 43: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 7 (female) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 8



Figure 44: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 8 (female) using primers P2 forward and reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results



Figure 45: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 8 (female) using Primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 9



Figure 46: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 9 (female) using Primers P2 forward and reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results



Figure 47: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 9 (female) using Primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 10



Figure 48: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 10 (female) using primers P2 forward and reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results



Figure 49: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 10 (female) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-20 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

In many cases, these experiments were repeated several times, with the same results being obtained; no reliable evidence of viral shedding was seen during the 30-day period after introduction of these animals into the MSRU. Table 5 below shows a summary of results of screening of animal introduced into the MSRU.

Table 5: Results of all screening performed on animals introduced into MSRU

- = Negative result of PCR test

Mouse Number	MHV PCR	No. of times PCR repeated
1	-	7
2	-	5
3	-	1
4	-	1
5	-	2
6	-	1
7	-	3
8	-	1
9	-	1
10	-	1

3.13 MHV detection in animals born in the MSRU

Three breeding pairs were then set up from randomly chosen animals from the introduced cohort. Litters were weaned at 21 days after birth and faecal sampling from the offspring conducted daily from 3 days after weaning (excluding weekends) for 22 days to determine if they had contracted MHV and for how long.

Mouse 1 (weanling)

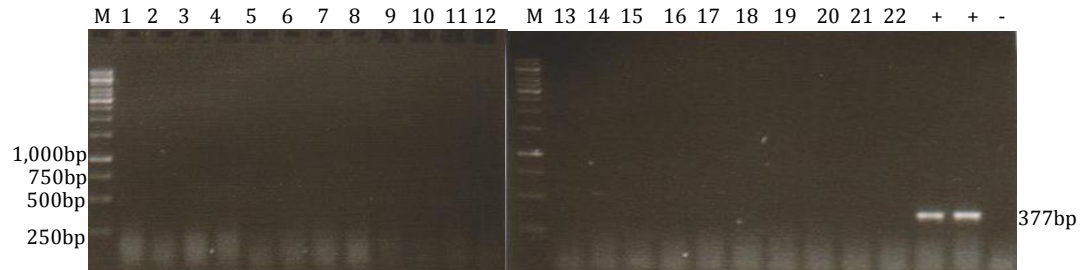


Figure 50: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 1 (weanling) using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results



Figure 51: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 1 (weanling) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 2 (weanling)



Figure 52: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 2 (weanling) using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

One positive result on day 6

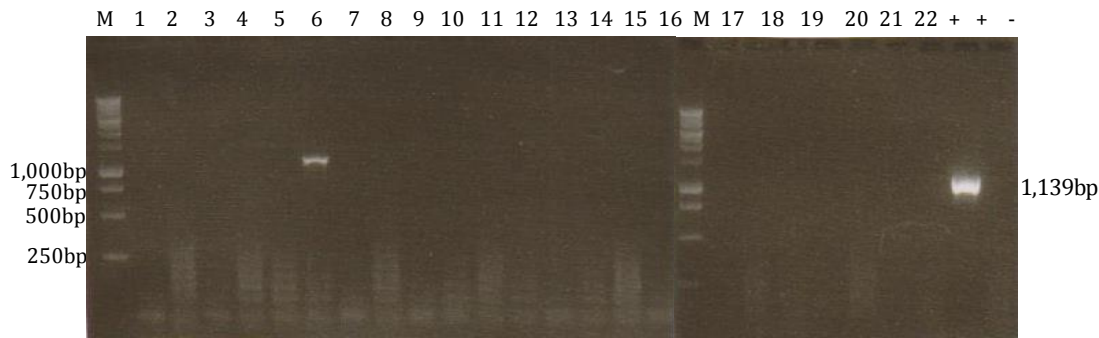


Figure 53: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 2 (weanling) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

One positive result on day 6

Mouse 3 (weanling)

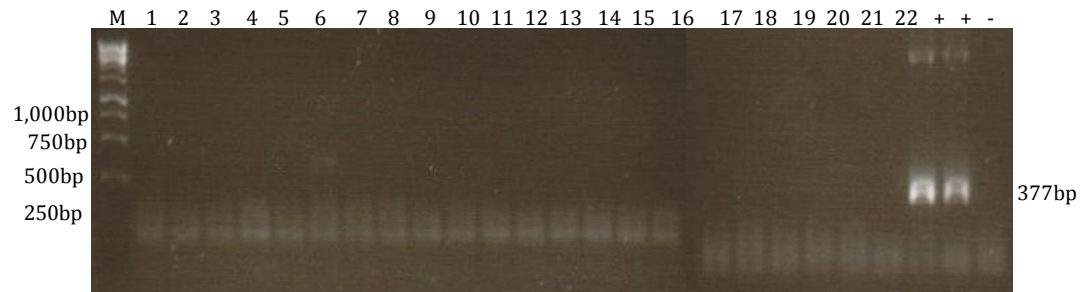


Figure 54: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 3 (weanling) using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

One positive result on day 6

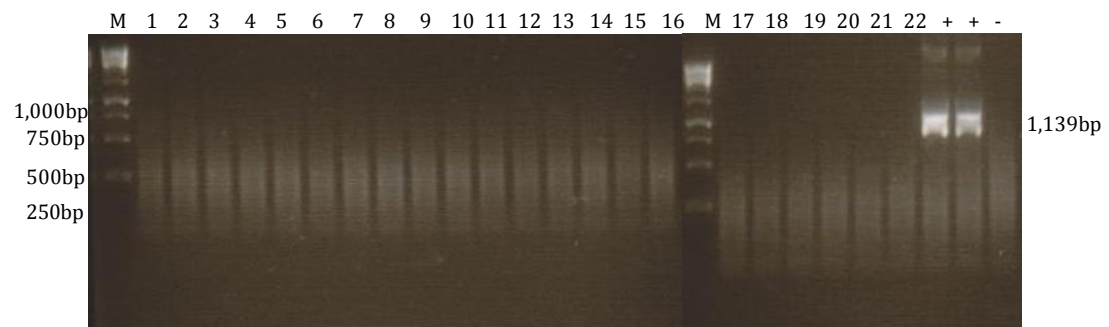


Figure 55: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 3 (weanling) using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 4 (weanling)



Figure 56: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 4 (weanling) using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

12 positive results from day 2 - 12 and day 22



Figure 57: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 4 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

5 positive results from day 2 – 6 and day 22

Mouse 5 (weanling)

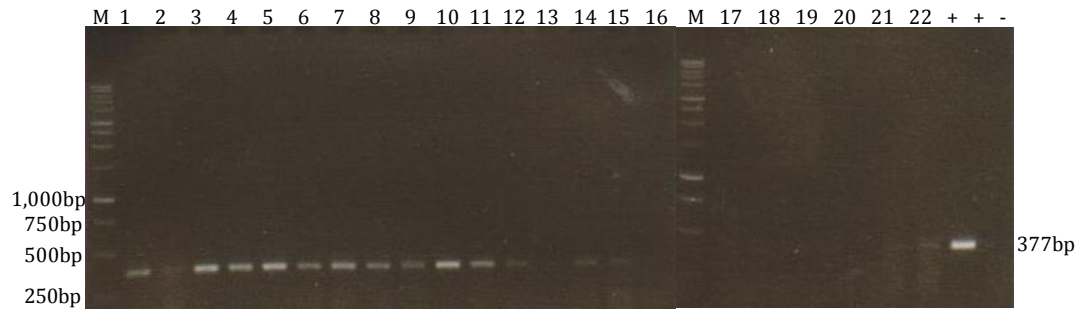


Figure 58: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 5 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

15 positive results from day 1 - 15



Figure 59: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 5 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 6 (weanling)

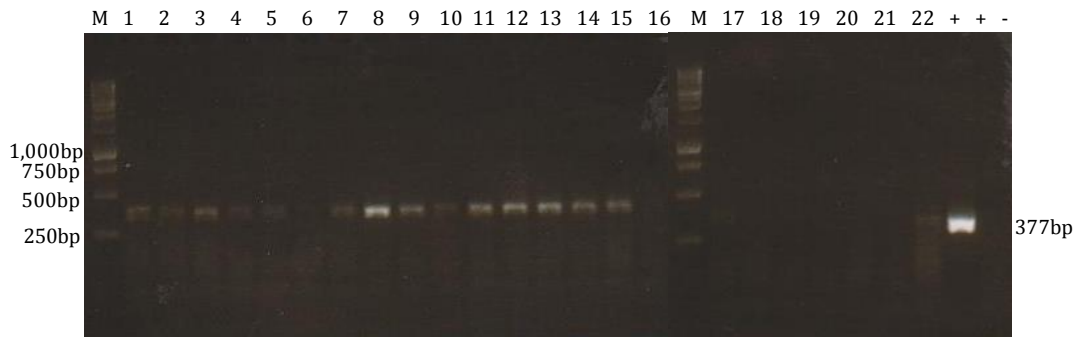


Figure 60: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 6 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

15 positive results from day 1 - 15

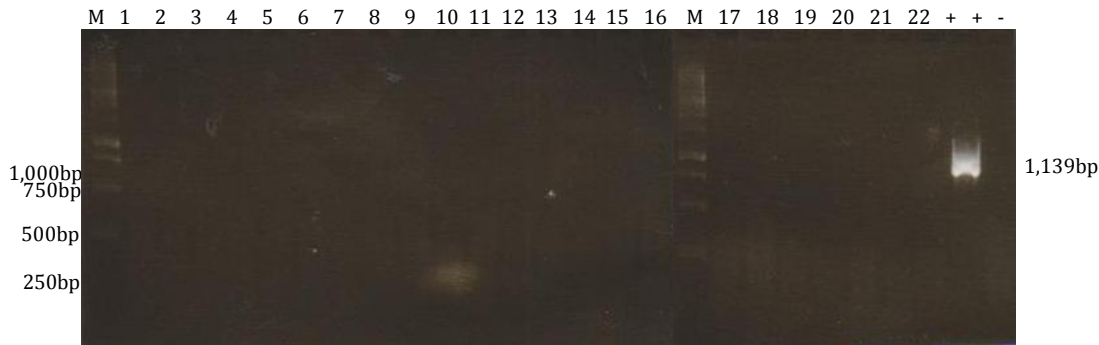


Figure 61: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 6 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 7 (weanling)



Figure 62: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 7 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

13 positive results from day 2 – 4, day 6 and day 8 - 16

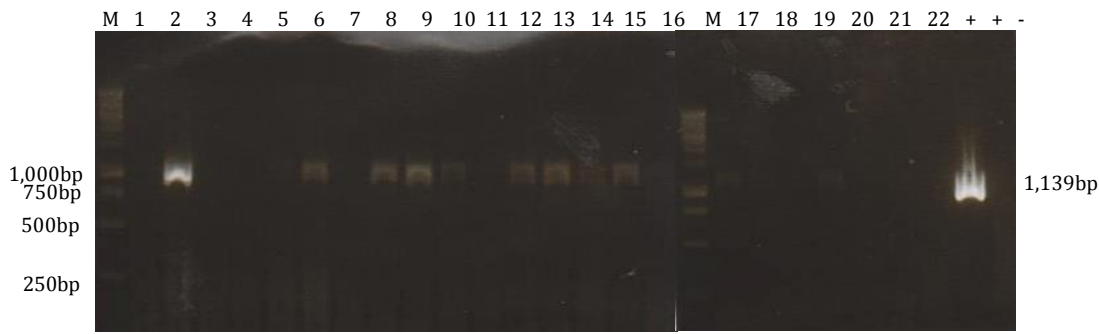


Figure 63: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 7 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

9 positive results on day 2,6, 8-10 and 12-15

Mouse 8 (weanling)

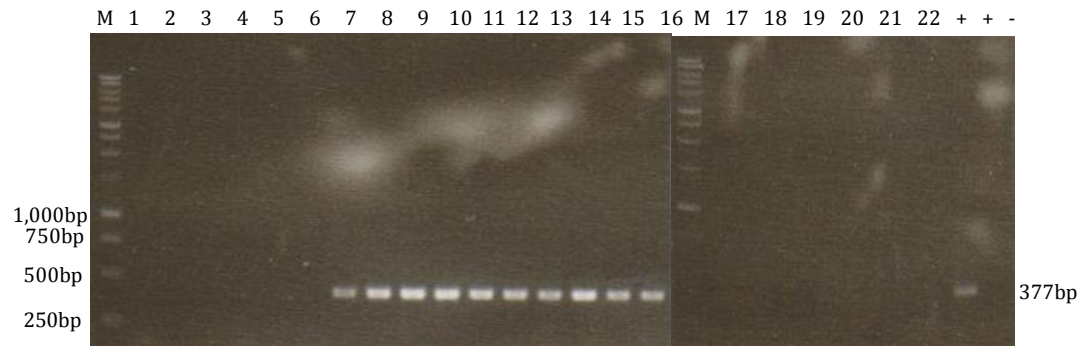


Figure 64: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 8 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

10 positive results from day 7 - 16

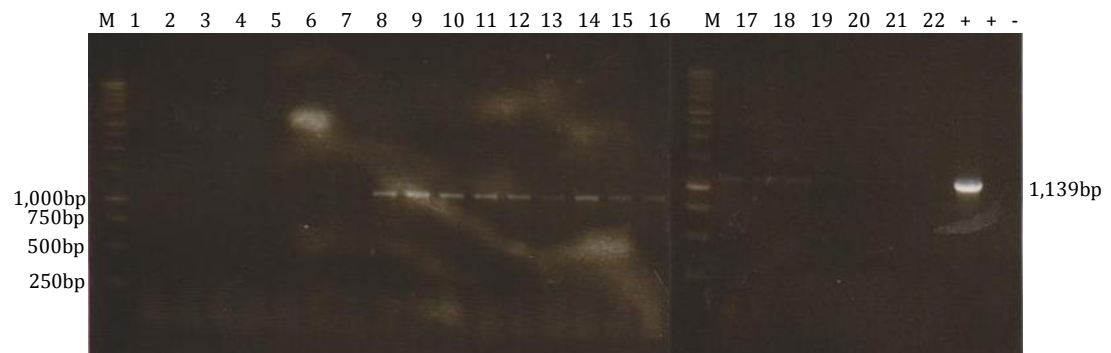


Figure 65: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 8 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

12 positive results from day 8 - 19

Mouse 9 (weanling)

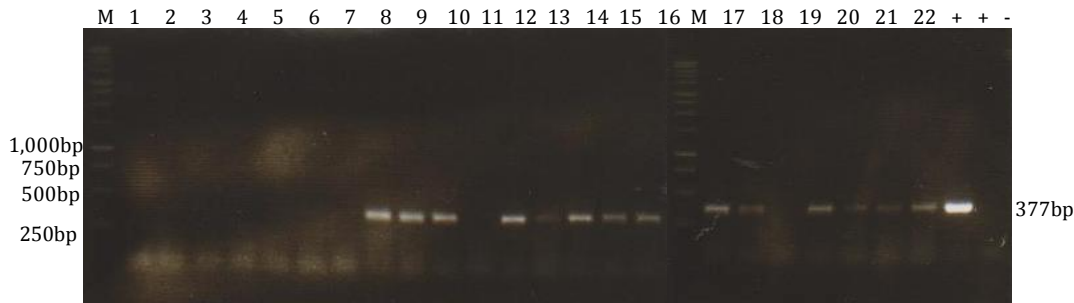


Figure 66: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 9 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

14 positive results from days 8 – 10, 12 – 18 and 20 - 22



Figure 67: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 9 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

6 positive results from days 8 – 10, 12, 13 and 17

Mouse 10 (weanling)



Figure 68: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 10 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

Positive results from day 1 – 6, 11 and 12, 16 and 17

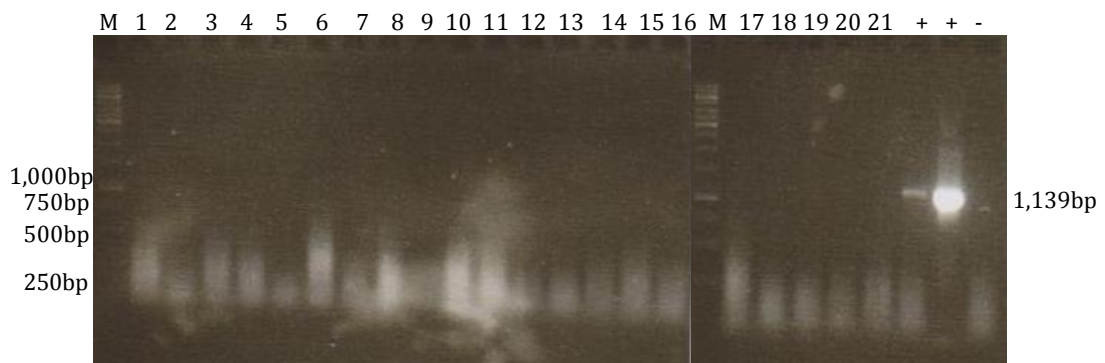


Figure 69: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 10 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 11 (weanling)



Figure 70: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 11 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

Positive results from day 2 - 4



Figure 71: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 11 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 12 (weanling)

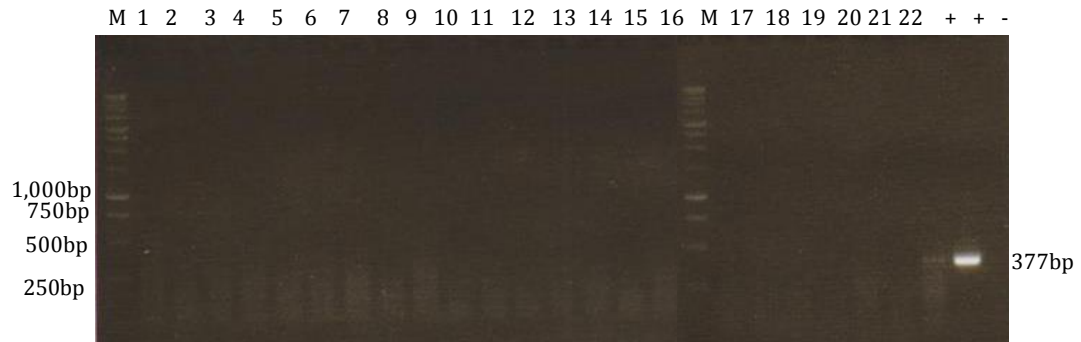


Figure 72: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 12 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

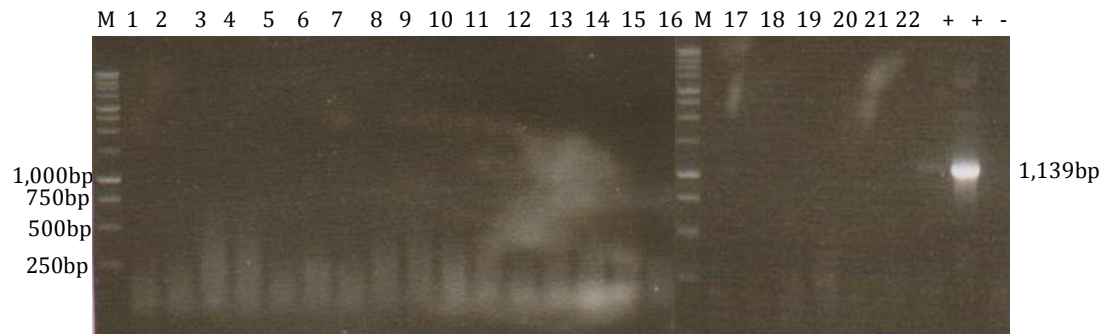


Figure 73: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 12 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 13 (weanling)

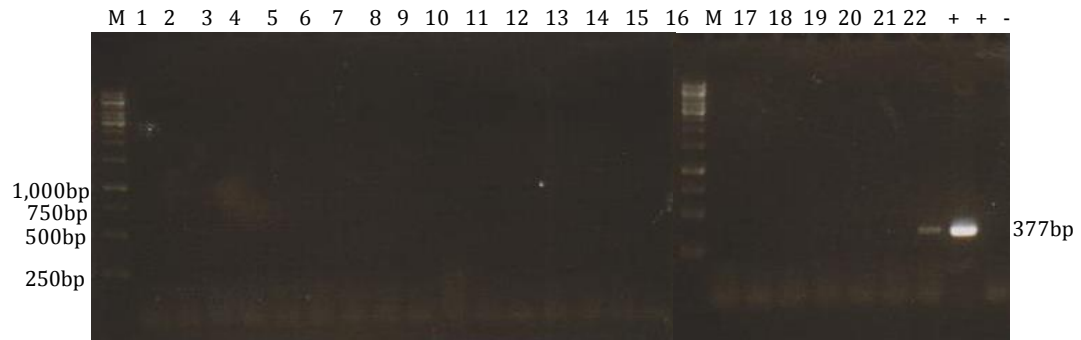


Figure 74: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 13 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

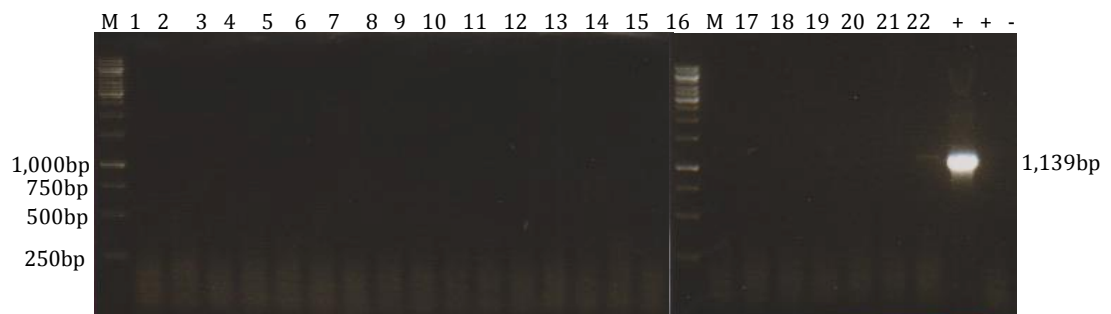


Figure 75: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 13 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 14 (weanling)

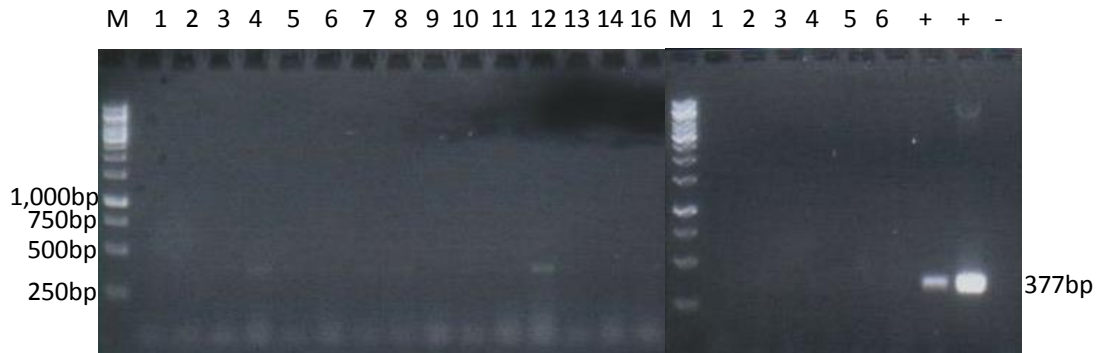


Figure 76: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 14 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

Positive result on day 4 and 12



Figure 77: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 14 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 15 (weanling)



Figure 78: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 15 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

Positive result on day 1 and 10

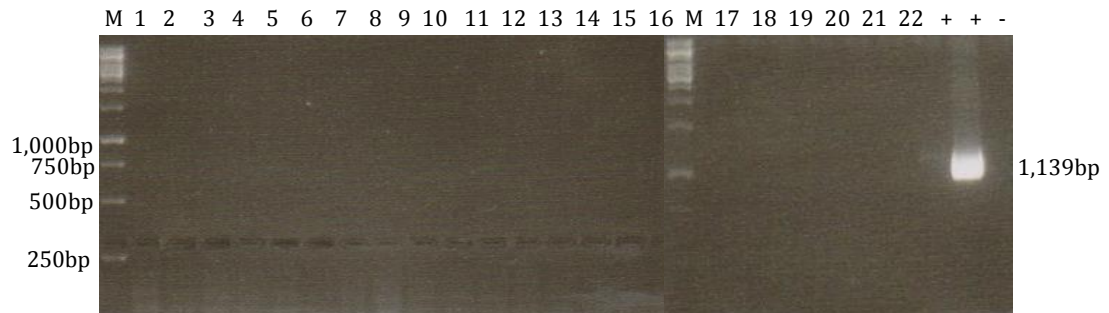


Figure 79: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 15 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

Mouse 16 (weanling)

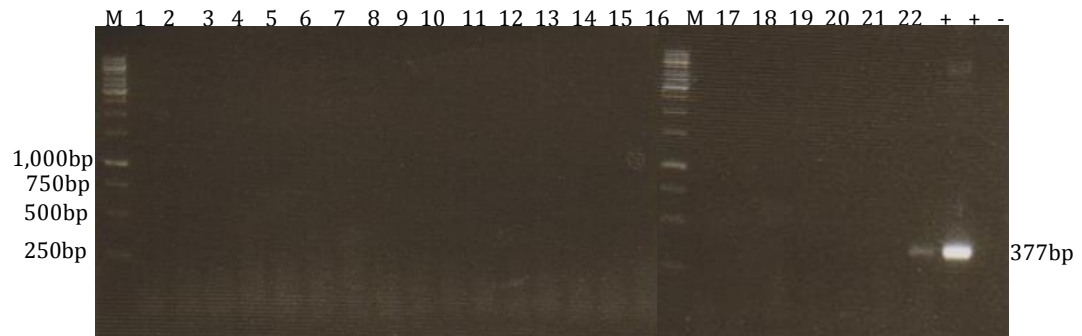


Figure 80: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 16 using primers P2 forward and reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

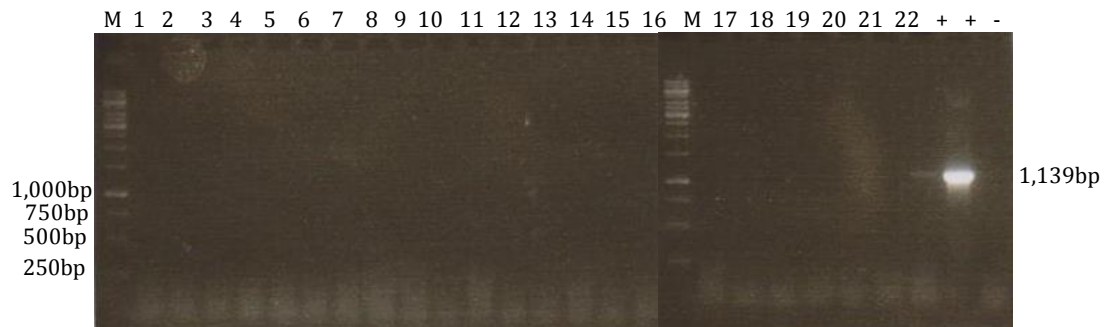


Figure 81: Agarose gel electrophoresis of total RNA and RT-PCR product of Mouse 16 using primers P1 forward and P3 reverse.

M = DNA marker ladder, 1-22 = days faecal samples were taken from mouse, first + = sample of known MHV positive mouse in MSRU, second + = synthetic positive control, - = negative control

No positive results

In summary, a range of different patterns were observed:

- Some animals never showed evidence of having become infected (Mouse 1, 12, 13 and 16).
- Some animals gave a reproducible viral signal on one or two days at some point during the observation period (Mouse 2, 3, 11, 14 and 15).
- Some animals appeared to be shedding virus over a prolonged period of time, including in some cases the last day of observation (Mouse 4, 5, 6, 7, 8, 9, 10).
- In some animals, there appeared to be more than one cycle of infection (Mouse 4, 7, 9, 10, 14 and 15).

In some of the mice (44%), one PCR gave a positive result and the other was apparently negative. In every case, the positive result was obtained when the smaller PCR fragment was being amplified. It is thus possible that amplification was more efficient in this reaction. In general, the smaller PCR worked well, with the band clearly distinct from the dye front on the gel (in which the primers are expected to run) and was therefore considered reliable. It is clear that there is not one simple pattern of infection. Table 6 shows the pattern of infection of 12 of the weanling mice, with 50% of these mice testing positive for both PCR and serology.

Table 6: Pattern of infection of 12 weanling mice

	+ PCR	- PCR
+ Serology	6	2
- Serology	3	1

3.14 Comparison of serology and PCR data

Blood samples were taken from 22 of the 26 mice and sent for serology testing to compare results with the RT-PCR to see which animals had in fact become infected with MHV.

Table 7: Results of Opti-Spot Serology Testing of 22 mice.

+ = positive, - = negative, W = weak positive, N = normal IgG

Mouse Number	Mouse IgG	MHV Serology	MHV PCR	Mouse selected as breeder
1	N	+	-	✓
2	N	W	-	✓
3	N	+	-	✓
4	N	-	-	x
5	N	W	-	x
6	N	+	-	✓
7	N	-	-	✓
8	N	+	-	✓
9	N	+	-	x
10	N	+	-	x
1 (weanling)	N	+	-	x
2 (weanling)	N	+	+	x
3 (weanling)	Not tested	Not tested	Faint band	x
4 (weanling)	N	+	+	x
5 (weanling)	N	+	+	x
6 (weanling)	Not tested	Not tested	+	x
7 (weanling)	N	+	+	x
8 (weanling)	N	+	+	x
9 (weanling)	Not tested	Not tested	+	x
10 (weanling)	N	-	+	x
11 (weanling)	N	-	Faint band	x
12 (weanling)	Not tested	Not tested	-	x
13 (weanling)	N	+	-	x
14 (weanling)	N	+	Faint bands	x
15 (weanling)	N	-	+	x
16 (weanling)	N	-	-	x

For animals 1-10 (those introduced into the MSRU), a serological positive was obtained in most, but not all, cases, even though no viral shedding had been detected in any of them. Note that the blood sampling took place several months after the experiment, so it is possible that these animals only became infected after the faecal sampling had stopped.

For the 12 sampled weanlings, eight were unequivocally positive by serology and four negative. Six animals were positive for both serology and the MHV PCR (weanlings 2, 4, 5, 7, 8 and 14) and one animal was negative for both tests (weanling 16). As with the adults, a pattern of positive serology but negative PCR results was seen in some animals (weanlings 1 and 13), presumably from the same cause(s). More surprisingly, three animals (weanlings 10, 11 and 15) that had given positive PCR results were negative for anti-viral antibodies when tested some months later. This suggests that, in some cases, animals can become infected and shed detectable amounts of virus, but do not develop a strong enough immunological “memory” for this latter to be observed in a serological test.

4 Discussion

4.1 Development of the PCR assay

The current method for detection of MHV and other diseases in the MSRU at Ninewells Hospital is to send blood samples for serology testing using sentinel animals. This study aims to address detecting a natural infection of MHV using a fast, easy and accurate way of testing in mouse colonies in-house by PCR. This study demonstrates analysis in mouse colonies through PCR of faecal matter. Previous methodology has limitations due to the infancy of PCR testing which has not been addressed.

A standard approach to the number of faecal samples to collect from each animal, and to their processing for RT-PCR has been developed. The general method was validated by checking for the detection of genes naturally expressed in mouse epithelial cells (fragments of which will appear in the faecal content). A synthetic positive control sequence was also commissioned and gave PCR products of the expected sizes.

Faecal samples were initially collected from 10 random weanling mice currently housed in various rooms in the MSRU at Ninewells hospital. This was done a number of times and initial results gave positives in about 30% of cases. This was consistent with the historical serological detection rate in the MSRU. Sequencing of a positive PCR product confirmed that the MHV N-gene was indeed being detected. One of these positive results was used as a positive control in subsequent experiments.

Initial experiments with a nested PCR to further amplify the signal appeared to be promising but, despite strenuous efforts to avoid cross-contamination, had a high rate of “false positives”, e.g., when water was added to the PCR reaction instead of the putative DNA sample. It was therefore decided to optimise a single-step PCR reaction (in terms of careful selection of primer sequences and the fine-tuning of temperatures).

Even so, there were a number of occasions on which significant cross-contamination occurred. It has to be acknowledged that the work took place in a laboratory routinely handling samples from mice from the MSRU and therefore that there would have been a fairly constant “background”. Nevertheless, when scrupulous care was taken to preserve the purity of each component of the PCR reaction, reproducible results could indeed be obtained.

No attempt was made in this project to quantify the viral load in the samples, results were simply classed as positive, negative or faint band by visual comparison with the relevant controls run on the same electrophoresis gel. There are a number of quantitative PCR methods available now (e.g. TaqMAN), but these were not necessary in order to draw the conclusions reached below.

Future work in the area of method development could include:

1. Revisiting the “one-tube” approach to RT-PCR, to try to make the assay more convenient, and possibly cheaper;
2. Moving all the analytical work to a laboratory off-site, when background contamination would be less of an issue; and reattempting the Nested PCR without using the synthetic positive control.
3. In-depth analysis of the sequences being amplified, in order to understand their homology to, and likely functional differences from, those viral sequences already in the databases.
4. Semi-quantitative PCR to measure viral copy number. This could be calibrated using the synthetic positive control DNA (though assumptions would still have to be made as to the efficiencies of extraction of RNA from the faecal pellets and its reverse transcription.
5. Targeting another highly conserved area of the viral genome i.e., M gene.

4.2 Viral detection in animals brought into the MSRU

Given the fairly high prevalence of the virus in the animal facility as a whole (around 30% by serology (see table 3) and my own initial PCR findings of randomly chosen weanling mice) and the housing of these ten animals in a heavily stocked mouse breeding room, we expected all the mice to become infected and to shed the virus within a short period of time. Other publications (Barthold, Beck et al. 1993) and (Casebolt, Qian et al. 1997) suggest that animals exposed to the virus should start shedding within a few days, though this was after direct inoculation.

To our surprise, none of the 10 mice showed evidence of shedding the virus at any point over the 30 days of sampling. The straightforward detection of MHV in the general population suggested that this was not a problem of insufficient sensitivity.

It may be that adult animals are much less susceptible, even though they have no specific pre-existing immunity to the virus, than weanlings are to infection. C57BL/6 mice have been claimed to be less susceptible (or at least likely to seroconvert) than other mouse strains to MHV (Matthaei, Berry et al. 1998), but a great majority of the mice in the MSRU are in this genetic background and yet MHV can routinely be detected in a significant minority by serology and PCR.

When the ten mice were killed some months later and samples taken for serological analysis, then the majority (80%), but not all, were reported as positive. We have to conclude that, in those animals, either the viral load never reached levels at which the PCR assay could detect it, or that infection first occurred sometime after the first 30 days of presumed exposure.

It thus appears that MHV may fail to cause a productive infection in adult C57BL/6J mice for several weeks after introduction, though it may infect them at some point thereafter. This means that the absence of a PCR-positive result cannot be taken immediately to mean the absence of the virus itself, but that testing might have to continue for several months. Some animals may not sero-convert at all, even after several months of exposure.

Future work in this area could include:

1. Extending the faecal sampling period from 30 days to 60-90 days, or even longer. Note that the sampling frequency would probably have to remain daily, as shedding, when it does occur, can be fleeting (see below);
2. Comparing the susceptibility of adult C57BL/6J mice with that of other inbred lines of laboratory mice (e.g., Balb/c);
3. Serial blood sampling for serology over the same observation period (this would require licensing under the Animals (Scientific Procedures) Act 1986.
4. Exposure of the animals to used bedding from other cages. While this might boost the infection rate, and is the standard practice when using "sentinel" animals in screening programmes, it is not representative of normal husbandry practices in animal units and would therefore not be informative as to the expected rates of transmission

4.3 Viral detection in animals born in the MSRU

Three sets of breeding pairs from the 10 mice were then set up to produce off-spring in order to perform further testing. There were 16 pups born and these were weaned after 21 days. They were housed with their litter mates of the same sex. Faecal samples were then collected from the first day after weaning for 22 days over a 30 day period.

MHV was detected in 12 (75%) of the 16 weanlings tested, at some point during the sampling period. However, a range of outcomes was observed. In some animals, viral shedding was observed on only one or two consecutive days. However, in others, the pattern was much more complex, with continued detection being seen in some and cyclical shedding in others.

There is no simple explanation of these findings. It may be that the animals still had variable levels of passive maternal immunity or they simply became exposed to “environmental” virus in different amounts and/or on different dates.

What is clear is that there can be no safe assumptions as to how long it may take for this particular strain of MHV to “burn out” in the MSRU.

Three animals tested positive for MHV by PCR but were then reported not to have seroconverted. Presumably, for some reason the immune response was neither strong enough to prevent the second cycle of infection in weanling 10 nor to produce long-lasting antibody levels in this animal or in weanlings 11 and 15. It is presently unclear why this should have happened.

Further work should include:

1. Isolation of mating pairs and then their offspring from the general environment, to determine whether offspring become infected primarily from their parents or from animals in adjoining cages;
2. Repetition of the experiments on a much larger scale, to determine just how many patterns of infection can be expected in animals born in the unit.
3. Application of semi-quantitative methods to determine whether “continuous” and “cyclical” patterns of infection are truly different phenomena.

4.4 Conclusion

In terms of the original aims of the project, it is indeed possible to develop a simple and rapid PCR assay that detects the strain of MHV circulating in the MSRU, however improved sensitivity of PCR may be needed. Previous assumptions as to the rate with which naïve animals can become infected and the period over which an infected animal can continue to shed the virus have been overturned. Much more work will be required before the feasibility of a “fire-break” approach to the eradication of this organism can be assessed. Serology, while generally accurate as to the historical exposure, is not 100% reliable. Improved sensitivity of the PCR may also be required.

References

- Adami, C., J. Pooley, et al. (1995). "EVOLUTION OF MOUSE HEPATITIS-VIRUS (MHV) DURING CHRONIC INFECTION - QUASI-SPECIES NATURE OF THE PERSISTING MHV RNA." Virology **209**(2): 337-346.
- Agersborg, S. S., K. M. Garza, et al. (2001). "Intestinal parasitism terminates self tolerance and enhances neonatal induction of autoimmune disease and memory." Eur J Immunol **31**(3): 851-9.
- Baker, D. G. (1998). "Natural pathogens of laboratory mice, rats, and rabbits and their effects on research." Clin Microbiol Rev **11**(2): 231-66.
- Barthold, S. W. (1986a). Research complications and state of knowledge of rodent coronaviruses. Washington, Hemisphere.
- Barthold, S. W., D. S. Beck, et al. (1993). Laboratory Animal Science **43**: 276-284.
- Barthold, S. W., D. S. Beck, et al. (1993). "Enterotropic coronavirus (mouse hepatitis virus) in mice: influence of host age and strain on infection and disease." Lab Anim Sci **43**(4): 276-84.
- Bergmann, C. C., T. E. Lane, et al. (2006). "Coronavirus infection of the central nervous system: host-virus stand-off." Nat Rev Microbiol **4**(2): 121-32.
- Bosch, B. J., R. van der Zee, et al. (2003). "The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex." J Virol **77**(16): 8801-11.
- Casebolt, D. B., B. Qian, et al. (1997). "Detection of enterotropic mouse hepatitis virus fecal excretion by polymerase chain reaction." Lab Anim Sci **47**(1): 6-10.
- Compton, S. R., S. W. Barthold, et al. (1993). "The cellular and molecular pathogenesis of coronaviruses." Lab Anim Sci **43**(1): 15-28.
- de Groot, R. J. (2006). "Structure, function and evolution of the hemagglutinin-esterase proteins of corona- and toroviruses." Glycoconj J **23**(1-2): 59-72.
- F R Homberger, A. L. S., and S W Barthold (1991). "Detection of rodent coronaviruses in tissues and cell cultures by using polymerase chain reaction." Journal of Clinical Microbiology **29**(12): 2789-93.
- Gilbert, S. F. (2013). Developmental Biology, Sinauer Associates Inc.
- Henderson, C. J., A. R. Cameron, et al. "Evidence that the capacity of nongenotoxic carcinogens to induce oxidative stress is subject to marked variability." Toxicol Sci **145**(1): 138-48.
- Homberger, F. R. (1997). "Enterotropic mouse hepatitis virus." Lab Anim **31**(2): 97-115.
- Homberger, F. R., L. Zhang, et al. (1998). "Prevalence of enterotropic and polytropic mouse hepatitis virus in enzootically infected mouse colonies." Lab Anim Sci **48**(1): 50-4.
- Homberger, F. R., L. N. Zhang, et al. (1998). "Prevalence of enterotropic and polytropic mouse hepatitis virus in enzootically infected mouse colonies." Laboratory Animal Science **48**(1): 50-54.
- Jacoby, R. O. and L. J. Ballgoodrich (1995). "Parvovirus Infections of Mice and Rats." Seminars in Virology **6**(5): 329-337.
- Karst, S. M., C. E. Wobus, et al. (2003). "STAT1-dependent innate immunity to a Norwalk-like virus." Science **299**(5612): 1575-8.
- Koressaar T, R. M. (2007). "Enhancements and modifications of primer design program Primer3." Bioinformatics **23**(10): 1289-91.
- Krzystyniak, K. and J. M. Dupuy (1984). Entry of Mouse Hepatitis Virus 3 Into Cells.

- Kunita, S., E. Terada, et al. (1992). "Sequence analysis and molecular detection of mouse hepatitis virus using the polymerase chain reaction." Lab Anim Sci **42**(6): 593-8.
- Kyriazis, A. P., L. DiPersio, et al. (1979). "Influence of the mouse hepatitis virus (MHV) infection on the growth of human tumors in the athymic mouse." Int J Cancer **23**(3): 402-9.
- Lai, M. M. (1990). "Coronavirus: organization, replication and expression of genome." Annu Rev Microbiol **44**: 303-33.
- Lai, M. M. and D. Cavanagh (1997). "The molecular biology of coronaviruses." Adv Virus Res **48**: 1-100.
- Leiter, E. H., P. H. Le, et al. (1988). "Genetic and environmental control of diabetes induction by multi-dose streptozotocin in two BALB/c substrains." Diabetes Res **9**(1): 5-10.
- Lorenz, T. C. (2012). "Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies." J Vis Exp(63): e3998.
- Mahler (Convenor), M., M. Berard, et al. (2014). "FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units." Lab Anim **48**(3): 178-192.
- Matthaei, K. I., J. R. Berry, et al. (1998). "Use of polymerase chain reaction to diagnose a natural outbreak of mouse hepatitis virus infection in nude mice." Lab Anim Sci **48**(2): 137-44.
- Nicklas, W., V. Kraft, et al. (1993). "Contamination of transplantable tumors, cell lines, and monoclonal antibodies with rodent viruses." Lab Anim Sci **43**(4): 296-300.
- Ning, Q., S. Lakatoo, et al. (2003). "Induction of prothrombinase fgl2 by the nucleocapsid protein of virulent mouse hepatitis virus is dependent on host hepatic nuclear factor-4 alpha." J Biol Chem **278**(18): 15541-9.
- Nuttall, P. A. and K. A. Harrap (1982). "Isolation of a coronavirus during studies on puffinosis, a disease of the Manx shearwater (*Puffinus puffinus*)." Arch Virol **73**(1): 1-13.
- Oyanagi, M., A. Kato, et al. (2004). "Detection of MHV-RNAs in mouse intestines and in filter dust in mouse room ventilation duct by a modified RT-nested PCR." Exp Anim **53**(1): 37-41.
- Pachuk, C. J., P. J. Bredenbeek, et al. (1989). "Molecular cloning of the gene encoding the putative polymerase of mouse hepatitis coronavirus, strain A59." Virology **171**(1): 141-8.
- Percy, D., Barthold SW, Ed. (2007). Pathology of Laboratory Rodents and Rabbits. Iowa, Blackwell Publishing Professional.
- Raamsman, M. J., J. K. Locker, et al. (2000). "Characterization of the coronavirus mouse hepatitis virus strain A59 small membrane protein E." J Virol **74**(5): 2333-42.
- Rao, G. N., W. W. Piegorsch, et al. (1989). "Influence of viral infections on body weight, survival, and tumor prevalence of B6C3F1 (C57BL/6N x C3H/HeN) mice in carcinogenicity studies." Fundam Appl Toxicol **13**(1): 156-64.
- Richter, C. B., J. E. Thigpen, et al. (1988). "Fatal pneumonia with terminal emaciation in nude mice caused by pneumonia virus of mice." Lab Anim Sci **38**(3): 255-61.
- Skinner, M. A. and S. G. Siddell (1983). "Coronavirus JHM: nucleotide sequence of the mRNA that encodes nucleocapsid protein." Nucleic Acids Res **11**(15): 5045-54.
- Smith, A. L., V. A. Carrano, et al. (1984). "Response of weanling random-bred mice to infection with pneumonia virus of mice (PVM)." Lab Anim Sci **34**(1): 35-7.
- Smith, A. L., R. O. Jacoby, et al. (1993). "In vivo studies with an "orphan" parvovirus of mice." Lab Anim Sci **43**(2): 175-82.

- Sturman, L. S., C. S. Ricard, et al. (1985). "Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments." J Virol **56**(3): 904-11.
- Taffs, L. F. (1976). "Pinworm infections in laboratory rodents: a review." Lab Anim **10**(1): 1-13.
- Uetsuka, K., H. Nakayama, et al. (1996). "Hepatitogenicity of three plaque purified variants of hepatotropic mouse hepatitis virus, MHV-2 in athymic nude mice." Exp Anim **45**(2): 183-7.
- Untergrasser A, C. I., Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012). "Primer3 - new capabilities and interfaces." Nucleic Acids Research **40**(15): e115.
- Virelizier, J. L., A. M. Virelizier, et al. (1976). "The role of circulating interferon in the modifications of immune responsiveness by mouse hepatitis virus (MHV-3)." J Immunol **117**(3): 748-53.
- Wang, R. F., W. L. Campbell, et al. (1999). "Diagnosis of mouse hepatitis virus contamination in mouse population by using nude mice and RT-PCR." Mol Cell Probes **13**(1): 29-33.
- Weir, E. C., D. G. Brownstein, et al. (1988). "Respiratory disease and wasting in athymic mice infected with pneumonia virus of mice." Lab Anim Sci **38**(2): 133-7.
- Weiss, S. R. and J. L. Leibowitz (2011). "Coronavirus pathogenesis." Adv Virus Res **81**: 85-164.
- Wilberz, S., H. J. Partke, et al. (1991). "Persistent MHV (mouse hepatitis virus) infection reduces the incidence of diabetes mellitus in non-obese diabetic mice." Diabetologia **34**(1): 2-5.
- Wunderlich, M. L., M. E. Dodge, et al. "Multiplexed fluorometric immunoassay testing methodology and troubleshooting." J Vis Exp(58).
- Yamada, Y. K., M. Yabe, et al. (1998). "Application of nested polymerase chain reaction to detection of mouse hepatitis virus in fecal specimens during a natural outbreak in an immunodeficient mouse colony." Exp Anim **47**(4): 261-4.
- Yokomori, K. and M. M. Lai (1991). "Mouse hepatitis virus S RNA sequence reveals that nonstructural proteins ns4 and ns5a are not essential for murine coronavirus replication." J Virol **65**(10): 5605-8.
- Yu, X., W. Bi, et al. (1994). "Mouse hepatitis virus gene 5b protein is a new virion envelope protein." Virology **202**(2): 1018-23.